SPECIFIC AND CROSS-REACTIVE ANTIBODY RESPONSE TO TYPHOID VACCINATION IN HUMANS

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ACADEMIC DISSERTATION

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ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I Pakkanen S, Kantele JM, Hedges S, Häkkinen M, Moldoveanu Z, Mestecky J, Kantele A. Expression of homing receptor on IgA1 and IgA2 plasmablasts in blood reflects differential distribution of IgA1 and IgA2 in various body fluids. Clinical and Vaccine Immunology 2010; 17: 393-40

II Pakkanen SH, Kantele JM, Kantele A. Cross-reactive gut-directed immune response against *Salmonella paratyphi* A and B in typhoid fever and after oral Ty21a typhoid vaccination. Vaccine 2012; 30: 6047-53.

III Kantele A,* Pakkanen SH,* Siitonen A, Karttunen R, Kantele JM. Live oral typhoid vaccine *Salmonella* Typhi Ty21a – a surrogate vaccine against non-typhoid *Salmonellae*? Vaccine 2012; 30: 7238-45.

IV Kantele A,* Pakkanen SH,* Karttunen R, Kantele JM. Head-to-head comparison of humoral immune responses to Vi capsular polysaccharide and Salmonella Typhi Ty21a typhoid vaccines – A randomized trial. PLoS One 2013; 8: e60583.

V Pakkanen SH, Kantele JM, Kantele A. Cross-reactive immune response induced by the Vi capsular polysaccharide typhoid vaccine against *Salmonella* Paratyphi strains – a randomized trial. Scan J Immunol 2014, Epub ahead of print Jan 2. doi: 10.1111/sji.12151.

VI Pakkanen SH, Kantele JM, Herzog C, Kantele A. Cross-reactive immune response against non-typhoid *Salmonellae* by parenteral Vi polysaccharide typhoid vaccine. Vaccine. 2014;32:544-551.

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* The authors contributed equally to the study.

ABBREVIATIONS

ALS	Antibodies in lymphocyte supernatant
APC	Antigen-presenting cell
ASC	Antibody-secreting cell
BSA	Bovine serum albumin
CI	Confidence interval
CLA	Cutaneous lymphocyte antigen
CMIS	Common mucosal immune system
dIg	Dimeric immunoglobulin
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
FCS	Fetal calf serum
GALT	Gut-associated lymphoid tissue
HEV	High endothelial venule
HR	Homing receptor
Ig	Immunoglobulin
iNTS	Invasive non-typhoid *Salmonella*
ISC	Immunoglobulin-secreting cell
J-chain	Joining-chain
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
M cell	Microfold cell
NTS	Non-typhoid *Salmonella*
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
pIg	Polymeric immunoglobulin
PP	Peyer's patch
SC	Secretory component
S-IgA	Secretory IgA
TAB	Parenteral whole-cell typhoid vaccine
Ty21a vaccine	*Salmonella* Typhi Ty21a vaccine
Vi vaccine	Vi capsular polysaccharide vaccine

ABSTRACT

Diseases caused by *Salmonella* (*S*.) comprise a serious health problem worldwide. *Salmonella* can cause enteric fever (typhoid fever and paratyphoid fever, caused by *S*. Typhi and *S*. Paratyphi A/B/C, respectively) or diarrhea (caused by non-typhoidal *Salmonella*, NTS serotypes). NTS serotypes can also cause severe invasive salmonellosis (iNTS). Typhoid fever is the only one of these diseases against which there are vaccines in clinical use: the oral live attenuated whole-cell vaccine *Salmonella* Typhi Ty21a (Ty21a vaccine) and the parenteral Vi capsular polysaccharide vaccine (Vi vaccine). Vaccines against paratyphoid fever and non-typhoid salmonellosis are urgently needed. Until a proper vaccine targeted against these diseases is in clinical use, a lower degree of the cross-protective capacity in currently available vaccines warrants evaluation. *S*. Paratyphi and numerous NTS serotypes are known to share antigens with *S*. Typhi. According to literature, one field trial and two retrospective studies with travellers have suggested cross-protective capacity for typhoid vaccines against paratyphoid fever.

This thesis examined the local and systemic antibody-mediated immune responses in volunteers immunized with typhoid vaccines and in patients with enteric fever. In addition to measuring *S*. Typhi-specific responses, this work addressed whether typhoid vaccines or natural typhoid fever have the capacity to elicit a cross-reactive immune response against other clinically relevant *Salmonellae* (paratyphoid or non-typhoid *Salmonellae*). Localization of the responses was characterized firstly by examining the homing properties of plasmablasts (systemic or intestinal homing) after vaccinations and in enteric fever and, secondly, by determining the typhoid-specific antibodies in IgA1 and IgA2 subclasses in various secretions after oral and rectal Ty21a vaccinations.

Orally and rectally administered Ty21a typhoid vaccines were both found to elicit a mucosal immune response in all secretions; in all of them, the typhoid-specific IgA1 exceeded IgA2. Comparisons between the immune responses to the two vaccines, Ty21a and Vi, revealed an equal magnitude of typhoid-specific plasmablasts, consistent with similar efficacy of these vaccines in field trials. The localization of the responses was different: Ty21a elicited a gut-directed

plasmablast response while the response to the Vi vaccine appeared more systemic.

The oral *Salmonella* Typhi Ty21a was found to induce a gut-directed plasmablast response cross-reactive with *S*. Paratyphi A and B and with numerous clinically significant non-typhoid *Salmonellae* (including the two most common ones, *S.* Enteritidis and *S.* Typhimurium). The response was higher against serotypes sharing two O-antigens (O-9,12) rather than only one O-antigen (O-12) with *S*. Typhi; no significant responses were seen against serotypes with discordant O-antigens. In animal experiments, intestinal antibodies against O-antigens have proved protective, and accordingly, our observation provides an immunological basis for the use of the Ty21a as a surrogate vaccine against paratyphoid and non-typhoid *Salmonellae*. A similar gut-directed cross-reactive plasmablast response was also observed in patients with natural enteric fever. Surprisingly, the Vi vaccine was found to elicit a systemic immune response cross-reactive with *S.* Paratyphi and the certain non-typhoid *Salmonellae*. This response was significantly weaker than with the Ty21a vaccine, presumably because it was only caused by contaminating lipopolysaccharide in the Vi vaccine preparation. In addition to plasmablasts, cross-reactive antibodies were found in the serum and in lymphocyte cultures in both vaccination groups.

The central finding of this thesis was that the Ty21a vaccine elicits a significant intestinal antibody-mediated immune response cross-reactive with *S.* Paratyphi and most clinically relevant non-typhoid *Salmonellae*. This finding is especially important in the present situation when vaccines against these pathogens are not available, the mortality rate for invasive NTS disease in Africa is high, and *S*. Paratyphi and NTS strains resistant to numerous antimicrobials are emerging around the world. Immunological evidence suggests that the currently available Ty21a vaccine could be used as a surrogate vaccine providing some cross-protection against paratyphoid A and B fever and numerous clinically important non-typhoid *Salmonellae*.

TIIVISTELMÄ

Salmonellataudit ovat vakava terveysuhka maailmanlaajuisesti. Salmonellabakteerit voivat aiheuttaa nk. enteerisen kuumetaudin (*S.* Typhi lavantaudin ja *S.* Paratyphi pikkulavantaudin) tai gastroenteriitin (nk. ripulisalmonellat, non-typhoidal Salmonellae, NTS); ripulisalmonellat voivat saada aikaan myös vakavaa invasiivista salmonelloosia (iNTS). Näistä ainoastaan lavantautia vastaan on saatavissa rokotteita: suun kautta annettava, eläviä heikennettyjä bakteereita sisältävä *Salmonella* Typhi Ty21a -rokote (Ty21a-rokote) ja intramuskulaarisesti annettava, puhdistettua Vi-kapselipolysakkaridia sisältävä valmiste (Vi rokote). Pikkulavantaudin aiheuttajilta ja NTS-serotyypeiltä suojaavaa rokotetta kaivataan kipeästi. Koska sellaista ei lähiaikoina liene tulossa kliiniseen käyttöön, kannattaa selvittää nykyisillä lavantautirokotteilla mahdollisesti saatava vähäinenkin ristisuoja. *S*. Paratyphillä ja monilla NTS-serotyypeillä tiedetään olevan rakenteellisia yhtäläisyyksiä *S*. Typhin kanssa. Kirjallisuuden mukaan yksi kenttäkoe ja kaksi retrospektiivistä matkailijatutkimusta osoittavat lavantautirokotteiden tarjoavan suojaa pikkulavantautia vastaan.

Tässä väitöskirjahankkeessa selvitettiin sekä paikallista (suoliston) että systeemistä vasta-ainevälitteistä immuunipuolustusta lavantautirokotteen saaneilla ja enteeristä kuumetta sairastavilla henkilöillä. *S*. Typhille spesifisten vasteiden lisäksi tutkittiin herättääkö lavantautirokote tai sairastettu lavantauti ristiin reagoivan immuunivasteen muita kliinisesti merkittäviä salmonelloja vastaan (pikkulavantaudin aiheuttajat ja NTS-serotyypit). Immuunivasteen paikantumista tarkasteltiin analysoimalla rokotuksen ja sairastetun taudin jälkeen plasmablastien kotiutumisprofiilia (kotiutuminen suoleen tai systeemiseen immuunijärjestelmään) sekä määrittämällä useista eritteistä *S*. Typhille spesifisten IgA1- ja IgA2-vasta-aineiden pitoisuuksia oraalisen ja rektaalisen Ty21a-rokotteen jälkeen.

Niin oraalisesti kuin rektaalisesti annetun rokotteen saaneilta todettiin kaikissa eritteissä mitata *S.* Typhille spesifinen immuunivaste. *S*. Typhi-spesifisen IgA1:n määrä osoittautui suuremmaksi kuin IgA2:n. Ty21a- ja Vi-rokotteilla saatiin yhtä voimakas *S*. Typhille spesifinen plasmablastivaste. Näiden rokotteiden suojateho on havaittu yhtäläiseksi myös kenttäkokeissa. Immuunivaste kuitenkin paikantui elimistössä eri tavoin: Ty21a rokote sai aikaan suoleen ohjautuvan vasteen, kun

taas Vi rokotteen vaste osoittautui systeemiseksi. Oraalinen Ty21a rokote herätti suoleen ohjautuvan, ristiin reagoivan plasmablastivasteen kahdelle merkittävimmälle pikkulavantaudin aiheuttajalle ja monille kliinisesti merkittäville NTS-serotyypeille (mm. yleisimmät serotyypit, *S*. Enteritidis ja *S*. Typhimurium). Vaste havaittiin voimakkaammaksi kannoille, joilla on *S*. Typhin kanssa kaksi yhteistä O-antigeenia (O-9, 12), kuin niille, joilla antigeenejä on vain yksi (O-12). Vastetta ei juurikaan syntynyt kannoille, joilla ei ole *S*. Typhin kanssa yhteisiä O-antigeenejä. Eläinkokeissa on osoitettu, että O-antigeenille spesifiset vasta-aineet suoliston alueella suojaavat niin gastroenteriitiltä kuin invasiiviselta salmonelloosilta. Tämän tutkimuksemme havainnot luovat immunologisen perustan Ty21a-rokotteen käytölle suojaamaan pikkulavantaudin aiheuttajia ja yleisimpiä NTS-tyyppejä vastaan. Samanlainen suolistoon ohjautuva, ristiin reagoiva immuunivaste todettiin myös enteeristä kuumetta sairastavilla. Yllättäen havaitsimme myös Vi-rokotteen herättävän ristiin reagoivan plasmablastivasteen pikkulavantaudin aiheuttajille ja samoille NTS-serotyypeille. Tämän ristireaktion aiheuttaa todennäköisesti rokotteen sisältämät lipopolysakkaridijäämät ja ristiin reagoiva vaste on huomattavasti matalampi kuin Ty1a-rokotteella aikaansaatu. Ristiin reagoivia vasta-aineita löytyi plasmablastivasteen lisäksi myös seerumista ja lymfosyyttiviljelmästä.

Väitöskirjatutkimuksen merkittävin havainto oli se, että oraalinen Ty21a-rokote voi synnyttää merkittävän ristiin reagoivan immuunivasteen pikkulavantaudin aiheuttajia ja kliinisesti merkittävimpiä NTS-serotyyppejä vastaan. Tämän tiedon arvo korostuu nykyhetken tilanteessa, jossa suojaavaa rokotetta ei ole saatavana, kuolleisuus invasiiviseen NTS-tautiin näyttää yhä lisääntyvän etenkin Afrikassa ja mikrobilääkkeille resistentit kannat yleistyvät sekä *S*. Paratyphi- että NTS-serotyyppien keskuudessa maailmanlaajuisesti. Immunologisten löydösten perusteella näyttää siltä, että nykyistä Ty21a-rokotetta voitaisiin käyttää korvaavana rokotteena antamaan ristisuojaa *S*. Paratyphi A:ta, B:tä ja monia kliinisesti merkittäviä NTS- serotyyppejä vastaan.

INTRODUCTION

Diseases caused by *Salmonella*, enteric fever and non-typhoidal salmonellosis comprise a serious health problem in major parts of the world. *Salmonella* serovar Typhi (*S*. Typhi) causes typhoid fever and *Salmonella* serovar Paratyphi A/B/C (*S.* Paratyphi A/B/C) paratyphoid fever, together known as enteric fever. Non-typhoidal *Salmonellae* (NTS) cause gastroenteritis and invasive non-typhoidal salmonellosis (iNTS). The estimated annual incidence of typhoid fever is 22 million,[1] paratyphoid fever 5.4 million,[2] and NTS diseases 94 million cases[3, 4] Collectively *Salmonella* infections are responsible for nearly 400 000 deaths per year, with 220 000 cases of typhoid and paratyphoid fever[2] and 155 000 cases of non-typhoid salmonellosis.[3]

The highest mortality and morbidity caused by *Salmonella* is seen in India, South-East Asia and Sub-Saharan Africa, in line with areas with a lack of clean water and adequate sanitation. In these low-income countries, *Salmonella* is one of the most common bacterial pathogens, being responsible for about half of all bacteremias.[5-7] Diseases caused by *Salmonellae* are also important in traveller[8] and especially non-typhoid salmonellosis, being one of the leading cause of foodborne illnesses, which constitute a significant problem in many high-income countries.[9-13] The emergence of strains resistant to antimicrobials complicates the situation even further.[14, 15] Typhoid fever is the only *Salmonella* disease against which there are vaccines in clinical use: the oral live attenuated whole-cell vaccine *Salmonella* Typhi Ty21a (Ty21a vaccine) and the parenteral Vi capsular polysaccharide vaccine (Vi vaccine). Vaccines against paratyphoid fever and non-typhoid salmonellosis are urgently needed. As long as there are no vaccines targeted against these diseases in clinical use, a lower degree of cross-protective capacity in preparations currently available warrants investigation. *S*. Paratyphi and many clinically important non-typhoid *Salmonella* have structures in common with *S*. Typhi (O-antigens 9 and/or 12). Previous and recent studies on the cell-mediated[16-18] and antibody-mediated[17-21] immune response to oral Ty21a vaccine suggest that there might be some cross-reactivity with individual *Salmonella* serotypes. Revisiting old data from field trials with the oral Ty21a vaccine revealed a protection rate of 49% against *S*. Paratyphi B by this vaccine.[22]

The local mucosal immune system in the intestine acts as a first line of defense against pathogens entering the human body via the oral route, such as *Salmonella*. Accordingly, the intestinal immune system has an important role in protection against salmonellosis. The immune mechanisms in natural salmonellosis or after Ty21a vaccination include both humoral and cell-mediated responses.[23-35] This thesis focuses only on antibody responses and mainly on O-antigen-specific responses.

1 REVIEW OF THE LITERATURE

1.1 B cells

The adaptive immune system comprises cell-mediated (T cells) and antibody-mediated parts (B cells).[36] This thesis focuses on the latter, B cells. Antibodies are proteins with 150-970 kDa molecular weight found in the serum and secretions, lymphatics and tissues of all vertebrates. They are produced by late-state B cell subsets, plasmablast and plasma cells. Antibodies have many important functions in preventing pathogens from causing disease.

Human B cells develop from hematopoietic stem cells mainly in the bone marrow.[37, 38] Each B cell produces antibodies that recognize their cognate antigen. Each individual has a multitude of B cells and can thus recognize numerous different antigens.[39] Immature B cells leave the bone marrow (primary lymphoid organ) to continue to circulate between secondary and tertiary lymphoid organs, also called peripheral lymphoid tissues, (e.g. Peyer's patches and lymph nodes) with the help of circulating blood and lymph.[40] They continue to patrol as naïve B cells until they encounter their cognate antigen in lymphoid tissues. They then become activated either by help of T cells (s.c. T cell-dependent immune response) or without T cells (s.c. T cell-independent immune response). Protein antigens are known to elicit a T cell-dependent response, whereas polysaccharide antigens activate a T cell-independent response.[37, 38] The activation leads to a maturation process of the B cells, with proliferation and clonal expansion of the cells, and differentiation to immunoglobulin-secreting cells (ISCs) or memory B cells, and affinity maturation and class-switching.[41] Activated cells leave the lymphatic tissue and travel via lymphatics and blood to secondary and tertiary lymphoid tissues (e.g. intestinal lamina propria). While migrating, B cells have already started to secrete antibodies specific to their antigen and they become antigen-specific antibody-secreting cells (ASC).[37, 38] These late-stage B cells are also called as plasmablast. After penetrating into the tissues to produce specific antibodies and the cells are known as plasma cells. Only a small portion of the circulating B cells secrete antibodies at any given time,[42] about 0.2-0.35% of peripheral blood mononuclear cells (PBMCs), representing less than 1% of all B cells.[43, 44]

1.2 Antigen-specific antibody-secreting cells (ASCs) and immunoglobulin secreting cells (ISCs)

The antigen-specific antibody-secreting cells (ASCs) are the cells that secrete antibodies against one particular antigen. ISCs represent the sum of all the ASCs present in the individual.[42] In this thesis, ISCs have been measured mostly as a positive control and to detect any possible previously undiagnosed immunodeficiencies, e.g. IgA deficiency (data not shown).

1.3 Circulation of B cells

While searching for their specific antigen, naive B cells are continuously recirculating in the body from lymphoid tissue to blood via lymphatics, the ductus thoracicus,[45] and then back to the lymphoid tissue.[46-50] The specific antigen is recognized by the naïve B cell and a part of the same antigen is presented to T cells by antigen-presenting cells (APCs) in lymph nodes or in the gut in the germinal center of Peyer's patches (PPs). Dendritic cells, as APCs, provide the B cells with information as to what kind of homing receptors (HRs) or chemokine receptors the B cells should express on their surface.[51-53] For example, in the presence of a vitamin A metabolite, the retinoic acid, in the gut, activate B cells to express receptors guiding them back to the gut ($\alpha_4\beta_7$-integrin and CCR9).[51-55] Dendritic cells and helper T cells guide B cells to differentiate to plasmablasts and initiate the secretion of antibodies.[51, 52] Antigen-specific plasmablasts, which are activated in PPs, migrate to the mesenteric lymph nodes. Afterwards, these cells as well as B cells activated in extraintestinal lymph nodes migrate via the lymphatics and thoracic duct to enter the blood circulation. Via blood they can spread throughout the body. Finally, they migrate to the tissues at the sites of the expected antigen encounter.[51, 52] (Figure 1.)

The antigen-specific plasmablasts can be studied in the peripheral blood during their migration in humans.[20, 23, 56-61] These cells appear in the peripheral blood 2-4 days after antigen encounter via mucosal [23, 58, 60, 62-67] and parenteral route.[57, 62] This has been demonstrated in various infections[64-66] as well as after vaccination.[64-66] as well as after vaccination.[57, 59-63, 68] The peak in the magnitude of ASCs is seen around day 7 following antigen encounter. They usually disappear gradually by day

14-16 after immunization.[23, 57, 62] The time period that these cells can be seen in blood are prolonged when exposure to the antigen is extended.[56, 69]

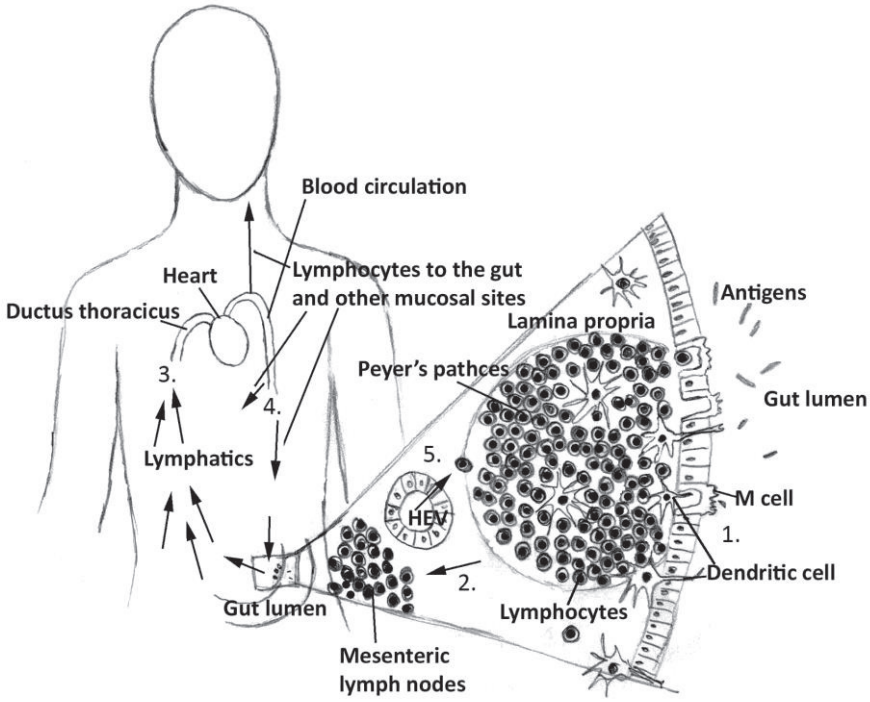

Figure 1. Antigen recognition in the gut and circulation of lymphocytes. 1. Antigen is ingested by M cells or by dendritic cells and presented to the lymphocytes in Peyer's patches in the lamina propria. 2. Activated lymphocytes migrate to the local lymph nodes; in the gut these are called mesenteric lymph nodes. 3. The activated lymphocytes migrate via lymphatic vessels and the ductus thoracicus to 4. blood circulation and 5. further to the site of the expected antigen encounter in the gut and other mucosal sites.

1.4 Homing of lymphocytes

Circulating lymphocytes, such as plasmablasts, are not distributed equally in the body, but they are guided to travel to the sites of expected antigen encounter. The homing of lymphocytes from blood into tissues is a multistep tissue-selective process. First, lymphocytes roll on specialized endothelial cells of post-capillary venules, called high endothelial venules (HEVs). (Figure 1.) Activation and

migration of lymphocytes occurs when their chemokine receptors recognize their specific ligand, chemokine (e.g. in the gut CCR9 binds CCL25) and their homing receptors (HRs) recognize their specific HR-ligand on the endothelial cells[40, 51, 52, 70] (see Figure 2).

Tissue-specific HRs include $\alpha_4\beta_7$-integrin, which guide the lymphocytes to the intestinal lamina propria,[71-73] L-selectin, which is associated with homing to the peripheral lymph nodes[74, 75] and cutaneous lymphocyte antigen (CLA), which guides cells to skin tissue.[76, 77] The selection of these HR and chemokine receptors on the lymphocytes reflects the expected target site of the immune effector cells in the body.

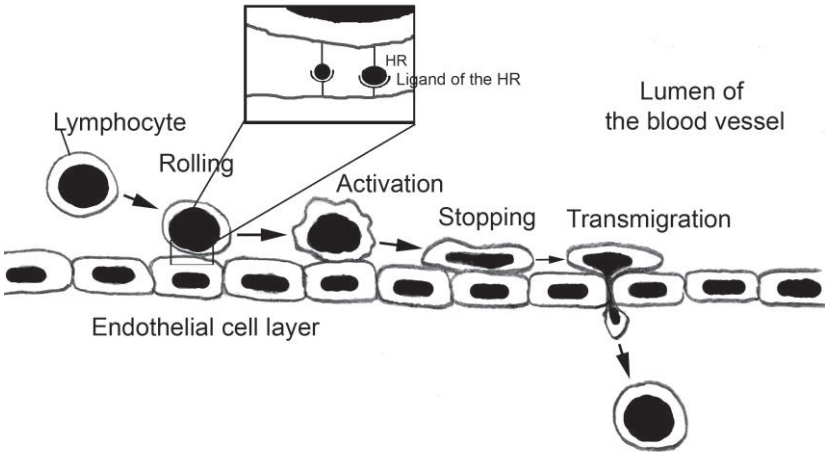

Figure 2. Homing of lymphocytes. When lymphocytes migrate via small specialized blood vessels, known as high endothelial venules, they contact the endothelial cell surface and begin rolling. Migration into the tissue occurs following ligand binding to the homing and chemokine receptors on the lymphocyte. The homing receptors bind to their ligands in the tissues, the lymphocytes become activated and then stop and migrate through the endothelial cell layer to the tissue. Modified from Kunkel & Butcher [40] and Salmi & Jalkanen[78]

1.4.1 α4β7-integrin

The $\alpha_4\beta_7$-integrin is known as the intestinal HR. It is located on the surface of the lymphocyte and guides the lymphocytes to the intestine by binding to its ligand, MAdCAM-1, on the HEV of intestinal lamina propria.[71] All plasmablast activated in the gut have been shown to express $\alpha_4\beta_7$-integrin.[79-81]

1.4.2 L-selectin

L-selectin (CD62L) is associated with guiding the cells to the peripheral lymph nodes,[74] but also to the inflamed tissue and the PPs.[82] In addition to its role as a systemic HR, L-selectin is expressed on naïve B cells, guiding them to secondary lymphoid organs.[51] It can bind several ligands, including the peripheral lymph node addressin (PNAd) and modified MAdCAM-1,[82] among many others.[83]

1.4.3 CLA

Cutaneous lymphocyte antigen (CLA) is a glycoprotein which serves as an HR, guiding the lymphocytes to the skin by binding the vascular lectin endothelial cell-leukocyte adhesion molecule 1 (ELAM-1).[76, 77, 84]

1.5 Immunoglobulins

Immunoglobulins (Ig) are Y-shaped molecules (monomeric Ig, mIg) with a molecular weight of about 150 kDa. The dimer and polymer forms of these Y-shaped molecules have molecular weights of 320 kDa and 620-970 kDa, respectively[85] Dimers and polymers are formed by two or multiple Y-shaped molecules, respectively, linking together with the joining (J) chain.[85] Secretory antibodies can bind a secretory component (SC), which has an important role when antibodies are transported to the mucosal site or to the secretions.[85]

Each of the Y-shaped molecules consists of two similar variable regions (in arms of the Y) and one constant region. The great variety in our antibody repertoire is made possible by the variable region of the immunoglobulin receptor gene that contains gene segments rearranged in multiple ways.[39] Antibodies that recognize the individual's own structures are mostly deleted during B cell development before leaving the bone marrow.[37] The constant region is similar in every antibody within a particular immunoglobulin class. There are five immunoglobulin classes: IgG, IgA, IgM, IgD and IgE. In humans IgG is divided into four subclasses IgG1-4 and IgA into subclasses IgA1 and IgA2.[85, 86] IgM-secreting cells can switch their Ig class from IgM to another isotype during their differentiation by recombination of genes

of the constant region.[38, 87, 88] To secrete mucosal IgA, this switching can take place after their arrival in the lamina propria[38, 87, 88] in the presence of DNA-editing enzyme activation-induced cytidine deaminase (AID) in B cells activated by T-independent antigens or by CD40/CD40L interaction in the presence of helper T cells and other signals such as interleukin 10 and 6 and transforming growth factor-β (TGF-β).[51, 89]

In the systemic and mucosal immune systems the Ig-isotypes are the same, but in serum IgA is mostly present as a monomer (mIgA) and in secretions as a dimer (dIgA) or polymer (pIgA). dIgA and pIgA are transported across the epithelium with the help of an SC receptor. After this transportation, part of this receptor, SC, remains bound to the dIgA/pIgA, thus constituting secretory IgA (S-IgA).[85] Because of the SC part, S-IgA is more resistant to proteolytic enzymes than any of the other Ig isotypes to proteolytic enzymes[85] In addition to IgA, also pIgM can bind SC.[85] Secretory IgM replaces S-IgA functionally in the majority of IgA-deficient individuals.[85]

Furthermore, the proportions of the various Ig isotypes are different in systemic fluids than in mucosal secretions. In the serum, most of the immunoglobulin belongs to the IgG isotype, and at the mucosal sites, IgA is the dominating isotype.[85, 90] Figure 3 illustrates the relative proportions of immunoglobulins in systemic fluids and mucosal secretions.

Antibodies in serum are mostly derived from B cells in the bone marrow and mucosal antibodies from B cells in local immune tissues. For example, antibodies in the gastrointestinal tract are secreted by B cells in the lamina propria of the gut.[85] The dIgA or pIgA and pIgM have been transported to the mucosal surfaces through epithelial cells by a special transport system, where SC has an important role. Other Igs are transferred to mucosal surfaces by an unspecific mechanism.[85]

Antibodies can protect an individual against pathogens and other harmful agents in multiple ways. They can, for example, block the invasion of pathogenic bacteria, neutralize viruses, toxins, or enzymes, opsonize microbes,[85, 91] and some isotypes can activate the complement system.[85, 91, 92]

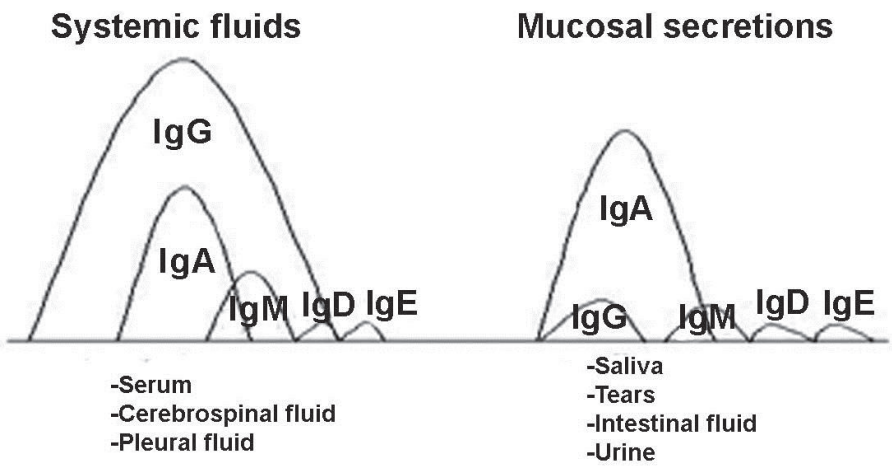

Figure 3. Immunoglobulins in the systemic fluids and mucosal secretions. The area under the curve illustrates the concentration of the particular immunoglobulin class. Modified from Kantele,[93] which is originally modified from Tomasi.[94]

1.5.1 IgA

The Peyer's patches of the gut are the principal source of IgA-producing B cells.[48] Dimeric IgA and pIgA are produced in Peyer's patches and mIgA by different IgA-producing cells mainly in systemic lymphoid tissues. The daily production of IgA in the body (66mg/kg/day) exceeds that of all other immunoglobulin isotypes combined and most of it is produced in the gut.[85, 90, 95] A daily amount of approximately 3 g of S-IgA is excreted into the gut lumen in adults.[95] IgA provides protection in several ways: S-IgA blocks the invasion of pathogenic bacteria, viruses and parasites by blocking their binding to epithelium receptors, capturing them in mucus, and assisting their removal by peristaltic and mucociliary activities.[96] This phenomenon is also known as immune exclusion.[97] In addition, IgA can neutralize viruses, toxins, or enzymes and inhibit the penetration of harmful antigens (such as carcinogens).[98-101] IgA activates complement only weakly.[92]

1.5.1.1 IgA1 and IgA2

In humans, IgA consists of two subclasses: IgA1 and IgA2. To avoid the protective effect of IgA, some pathogenic bacteria (e.g. *Haemophilus influenzae*, *Neisseria*

meningitidis, *Neisseria gonorrhoeae, Streptococcus pneumoniae*) produce proteases that destroy IgA1.[102, 103] However, this protease has no effect on IgA2.

IgA1 and IgA2 are unequally distributed in the body fluids. In serum or plasma, about 84% is IgA1, while only 16% is IgA2.[85] IgA1 dominates in all secretions, except intestinal and genital fluids, where the proportion of the IgA2 equals that of IgA1 or even dominates.[85, 104] The differential distribution of the IgA subclasses in secretions has been shown to be accompanied by a similar distribution of IgA1- and IgA2-producing cells at those sites.[85, 104-106] A higher proportion of IgA1 than IgA2 cells is found in the bone marrow, the respiratory tract, lacrimal glands, and the upper parts of the gastrointestinal tract, such as salivary glands and the small intestine, while IgA2 cells dominate in the lower part of the gastrointestinal tract.[105, 106] Immunization with polysaccharide vaccines elicits in both serum and ASCs an IgA2-dominated response,[104, 107, 108] while an IgA1-dominated response against the toxin protein is seen with both the whole-cell cholera vaccine and the natural cholera disease.[109]

1.6 Mucosal immunology in the gut

In the intestine, the mucosal immune system has the challenging mission of protecting the individual against pathogens over an area covering at least 400 m^2 while at the same time permitting commensal microbiota to grow in moderate proportions and allowing the intestine to function in its primary role: food absorption. The local mucosal immune system functions independently yet interacts with the systemic immune system. The majority of lymphocytes in the human body (80%) are located in the gut[53, 110] IgA, the major Ig in the gut, plays an important role as the first immunological defense barrier in the body since the mucosal sites act as a portal of entry for most human pathogens.[53, 101]

1.6.1 Mucosa-associated lymphoid tissue (MALT)

Human mucosa-associated lymphoid tissue (MALT) consists of several scattered cells and different immune tissues, which are located in distant parts of the human body.[111] MALT contains bronchus-associated lymphoid tissue (BALT), nasal-associated lymphoid tissue (NALT), gut-associated lymphoid tissue (GALT), and

lymphoid tissues in, for example, the genital tract and mammary and salivary glands.[111, 112] These different parts communicate with one another via circulating lymphocytes, collectively constituting the common mucosal immune system [113, 114] (see Section 1.3). However, the mucosally activated lymphocytes are not distributed by circulation equally to the various mucosal sites, instead favoring some sites above others. This phenomenon is called compartmentalization. For example, plasmablasts activated in the gut mainly migrate not only back to the gut but also to some other sites, mostly salivary and mammary glands.[113]

1.6.2 Gut-associated lymphoid tissue (GALT)

In humans, GALT comprises hundreds of Peyer's patches (PPs), small intestinal isolated lymphoid follicles, the appendix, and colonic lymphoid follicles.[115] Lamina propria and gut epithelium are not actual lymphoid tissues, but they are filled with immune cells because immune responses are expressed everywhere in the gut, not just in the lymphoid tissues.[115] PPs are organized areas of lymphoid tissue, which are covered by the follicle-associated epithelium, a specialized lymphoepithelium with microfold cells (M cells) and without crypts or villi. M cells are specialized to antigen ingestion and presentation.[38, 112] Below the epithelium is a dome region, with dendritic cells, B and T cells, and macrophages, below which are germinal centers, in which antigens are presented to the B cells.[112, 115] Large numbers of B cells are located in the germinal centers.[115] PPs are surrounded by high endothelial venules, expressing MAdCAM-1,[115] the ligand for lymphocyte intestinal HR, $\alpha_4\beta_7$ (see Sections 1.4 and 1.4.1).

1.7 Method for investigating antibody response after mucosal and parenteral immunization in humans

Antibody-mediated immune response to vaccination or infection can be explored by detecting antibodies in tissues, by measuring antibody concentrations in serum or various secretions, or by investigating circulating antibody-secreting cells. Antigen-specific plasmablasts, activated recently at either mucosal or systemic sites, can be detected in peripheral blood for a short period before they home to their effector site (see Sections 1.3 and 1.11.1). These plasmablasts can be

investigated by ELISPOT or by analyzing antibodies in supernatants of lymphocyte culture. ELISPOT is the most sensitive of these methods, as it detects the response at a single-cell level. The principle of the ELISPOT assay is illustrated in Figure 4. The ELISPOT assay for detecting vaccine-specific plasmablast or memory B cells is used in numerous studies to evaluate response after immunization with mucosal[19, 23, 26, 67, 80, 116-120] or parenteral vaccine.[57, 62, 80, 81, 121, 122]

The magnitude of the *S*. Typhi-specific ASC response in the ELISPOT assay in volunteers receiving the oral *Salmonella* Typhi Ty21a vaccine[23, 118] has been shown to correlate with the protective efficacy of the same vaccine regimen in field trials[118, 123-126] (see Section 1.11.5).

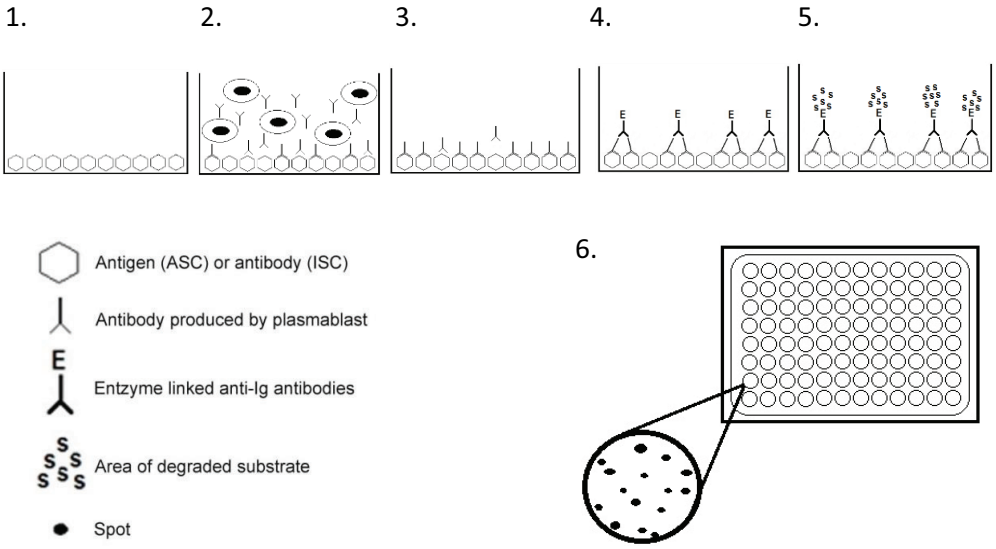

Figure 4. The principle of the ELISPOT assay.
Picture is drawn by Minna Vuojolainen, modified from Kantele.[23]

1. The wells are coated with the antigen/coating antibody (2h at 37 °C or overnight at 4°C), washed with PBS-Tween (0.05 %) and blocked with 1% BSA-PBS solution for at least 30 min at +37 °C and washed with PBS before adding the cells.
2. Isolated PBMC are incubated in the wells for 2-3h at +37 °C.
3. The cells and unbound antibodies are washed a away with PBS-Tween (0,05 %).
4. The wells are incubated with enzyme linked secondary antibody (2h at +37 °C or overnight at 4°C).
5. The substrate is added in hot agarose, which hardens quickly.
6. The locations where the plasmablasts once were are seen as colored spots. Each spot is considered to represent an imprint of a single ASC or ISC. The spots are calculated by a microscope or by the ELISPOT reader.

1.8 Salmonella

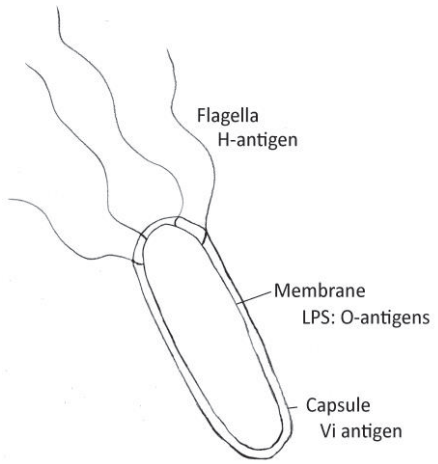

Figure 4. Simplified structure of *Salmonella* showing the flagellar H-antigen, capsular Vi antigen (expressed only in some *Salmonella* serotypes) and O-antigens of the lipopolysaccharide (LPS).

Salmonellae usually enter the host through the oral route via contaminated food or water. They are rod-shaped, Gram-negative, flagellated, facultative anaerobes belonging to the family *Enterobacteriaceae*.[127] (Figure 5). They are intracellular bacteria whose survival within macrophages is essential for their virulence.[128] The genus *Salmonella* consists of two species: *S. enterica* and *S. bongori* and more than 2500 serotypes. Serotypes belonging to *S. enterica* can cause diseases in humans. *S. enterica* is divided into the following six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae,* and *indica*.[129, 130] The subspecies are classified into serogroups based on the somatic O-antigens and divided into serovars based on the flagellar H antigen. The nomenclature, initially devised by Philip Bruce White and Fritz Kauffmann, is known as the Kauffmann–White classification.[130] The nomenclature of *Salmonella* serotypes has been revised: for example, *Salmonella enterica* subspecies *enterica* serovar Typhimurium should be abbreviated *S. enterica* subsp. *enterica* ser. Typhimurium.[131]However, similarly to the present thesis, the current literature mostly uses the shorter term *Salmonella* Typhimurium or *S*. Typhimurium.

S. enterica subsp. *enterica* contains typhoidal serotypes *S. enterica* subsp. *enterica* serotype Typhi (*S*. Typhi) and *S. enterica* subsp. *enterica* serotype Paratyphi A, B, and C (*S*. Paratyphi A, B, and C) causing enteric fever (typhoid and paratyphoid fever, respectively), while non-typhoid *Salmonella* (NTS) serotypes cause gastroenteritis, self-limiting diarrhea, and invasive non-typhoidal salmonellosis.[129] The clinical outcome of the *Salmonella* infection depends on many factors including serovar, host species, infecting dose, immunological competence, and gut flora of the host.[34] Outcome of the disease ranges from asymptomatic carriage to fatal systemic febrile illnesses.[34]

Table 1. Some of the most common *Salmonella enterica* subspecies *enterica* serotypes isolated in human samples, their O- and Vi antigen(s) and the clinical picture of the ensuing disease. Antigens present in the Ty21a vaccine are marked in bold.

Serotype	O- or Vi-antigen	Disease
Typhi	**9**, **12**, Vi	typhoid fever
Paratyphi A	1, 2, **12**	paratyphoid fever
Paratyphi B	1, 4, 5, **12**	paratyphoid fever
Enteritidis	1, **9**, **12**	gastroenteritis, iNTS
Typhimurium	1, 4, 5, **12**	gastroenteritis, iNTS
Dublin	**9**, **12,** Vi	gastroenteritis, iNTS
Stanleyville	4, **12**	gastroenteritis, iNTS
Heidelberg	1, 4, **12**	gastroenteritis, iNTS
Javiana	**9**, **12**	gastrioenteritis, iNTS

1.8.1 *Salmonella* Typhi

The causative microbe of typhoid fever is *S*. Typhi.[132] It causes disease only in humans.[132] Typhoid fever is a systemic disease manifesting with high fever and headache. Intestinal symptoms vary; stomach ache is common and may be accompanied by diarrhea or constipation. Most of the symptoms, including stupor, malaise, loss of weight, and death, at least in animal models, are caused by the recently identified typhoid toxin,[133, 134] which is only produced in mammalian cells.[135, 136] Typhoid toxin consists of two A subunits and one B subunit, which is homologous to one of the components of the B subunit of pertussis toxin. Subunit B has been demonstrated to contribute to the acute symptoms of typhoid fever.[133]

S. Typhi can migrate to the gall bladder and persist there throughout an individual's life, causing chronic asymptomatic carriage; such carriers may easily transmit the disease. Chronic carriage occurs in 2-5% of cases even after drug therapy.[8] The case fatality rate of typhoid fever has been 10-20% when microbial treatment was not available.[137] Antimicrobial treatment can be lifesaving, and with

use of appropriate drug therapy the case fatality rate is only 1%.[137] The world case fatality rate is 1-4%.[138] However, the widespread emergence of strains resistant to antimicrobials complicates this situation.[14, 138]

Globally, an estimated 21-22 million cases and more than 200 000 deaths due to typhoid fever occur annually.[1, 138] *S*. Typhi is transmitted by fecally contaminated food or water and by human-human contact by fecal oral route;[139] therefore it is most prevalent in places lacking clean water and adequate sanitation.[8, 25] The major burden of the illness is experienced by infants, children, and adolescents in South-Central and Southeastern Asia.[1, 140] Furthermore, typhoid fever is also an important travel-associated disease.[8]

1.8.2 *Salmonella* Paratyphi

Paratyphoid fever is caused by *S.* Paratyphi A and B and rarely by *S.* Paratyphi C. It is a systemic disease with clinical features indistinguishable from typhoid fever.[2, 141-145] Globally, an estimated 5.4 million cases of paratyphoid fever occur annually,[1, 2] with a higher incidence in endemic areas[8, 144, 146-148] and among travellers.[8, 144, 149] The major burden of paratyphoid fever is in Africa, Asia, and Latin America,[2, 150, 151] with the highest incidence in the Indian subcontinent and in south-east Asia.[2, 145, 150, 151] As with *S.* Typhi, there is serious concern about increasing antimicrobial resistance (resistance to nalidixic acid, decreased susceptibility to Ciprofloxacin, and Ceftriaxone or multidrug resistance) among *S.* Paratyphi strains.[144, 150-155] No vaccines against paratyphoid fever are currently available.

1.8.3 Non-typhoidal *Salmonella*

Non-typhoid *Salmonella* (NTS) is one of the leading causes of foodborne illnesses in both developing[156] and developed countries.[9-13] The estimated number of cases varies from 93.8 million[3] to 1.3 billion[4] with 155 000 deaths globally each year; of these 80.3 million cases are foodborne.[3] While the major burden of NTS worldwide constitutes infections restricted to the gastrointestinal tract, the morbidity to invasive NTS (iNTS) is high. Fever is the most common clinical feature in iNTS, and it can occur without gastrointestinal symptoms.[157, 158] The bacteria can become

distributed to various organs and cause e.g. septic arthritis.[158] Gastroenteritis caused by NTS is usually self-limiting and does not require microbial drug therapy, yet in iNTS disease appropriate drugs can be life-saving. However, increasing antimicrobial resistance and the emergence of multiresistant strains among NTS pose a serious problem for public health.[15, 159, 160]

Salmonella cause less than 1% of bacteremia in high-income countries.[161] Nevertheless, foodborne pathogens like NTS, constitute an increasing problem in high-income countries due to industrialization of food production.[162] In the USA, foodborne pathogens cause 1.4-9.4 million infections (confirmed-estimated), 15 000-55 961 hospitalizations and 400-1 351 deaths annually.[9, 10, 13, 163] Most of these cases are of domestic origin.[164] *Salmonella* is one of the most common confirmed cause of all foodborne infections in the USA, [9-13] accounting for 30% of the infections.[9, 13, 165] NTS causes 41 930 reported and 1 million estimated cases in USA annually.[10] It is the leading cause of foodborne hospitalizations and deaths (estimated 35% and 28%, respectively).[10] In low-income countries, NTS is among the most common bacterial pathogens.[6] In Africa, it has emerged as a significant cause of bloodstream infections, especially in children under two years of age or those with malaria or malnutrition or in adults with HIV infection.[6, 7, 156, 166, 167] iNTS has been shown to have a different clinical outcome in Africa than elsewhere; iNTS without diarrhea is common. The genome of the *S.* Typhimurium serotype in invasive infections in sub-Saharan Africa has been reported to have more similarities with *S*. Typhi than with *S*. Typhimurium found in other parts of the world.[168] This may explain the exceptional clinical outcome in Africa.[168]

The majority of NTS salmonellosis cases are caused by *S.* Enteritidis and *S.* Typhimurium worldwide.[129, 156, 157, 165, 169, 170] They represent 75% of the *Salmonella* cases in the EU[169] and 32% of all *Salmonella* isolates in USA (*S*. Enteritidis 17% and *S*. Typhimurium 15%).[129] Furthermore, most of the invasive NTS diseases in Africa are also caused by *S.* Enteritidis and *S.* Typhimurium.[156, 157]

1.8.4 Antigenic structures of the *Salmonella*

Various *Salmonella* serotypes express different antigenic structures, such as membrane proteins, porins, core regions and O-antigens of the lipopolysaccharide

(LPS), flagellar H-antigen and Vi antigen.[171, 172] This thesis focuses on the typhoidal antigens: O-9,12, H-d and V antigen. LPS is a component of the outer membrane of Gram-negative bacteria, and O-antigens are the outermost component of the LPS (Figure 6A). LPS contains also lipid A and a core polysaccharide (Figure 6B).

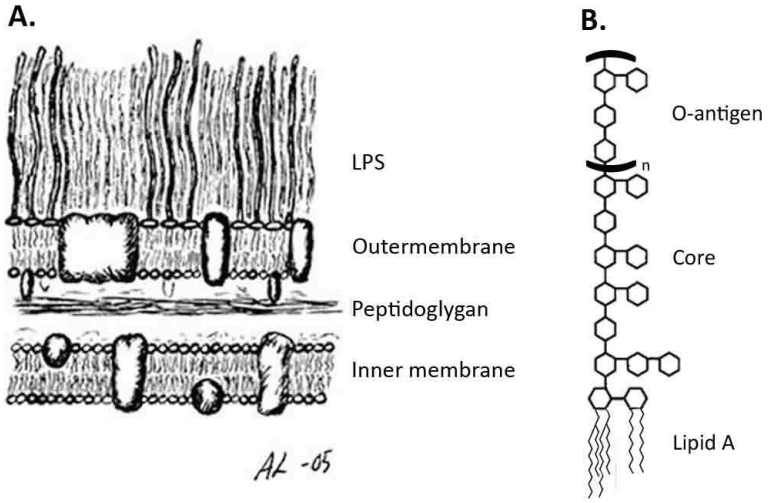

Figure 5. Systematic structure of the cell wall of Gram-negative bacteria and LPS. A) Cell wall of Gram-negative bacteria. Picture is drawn by Antti Lavikainen, first published by Meri.[173] B) LPS contains lipid A, core and O-antigen structures. O-antigen consists of multiple similar polysaccharide parts known as repeating units. Modified from Steinbacker et al.[174]

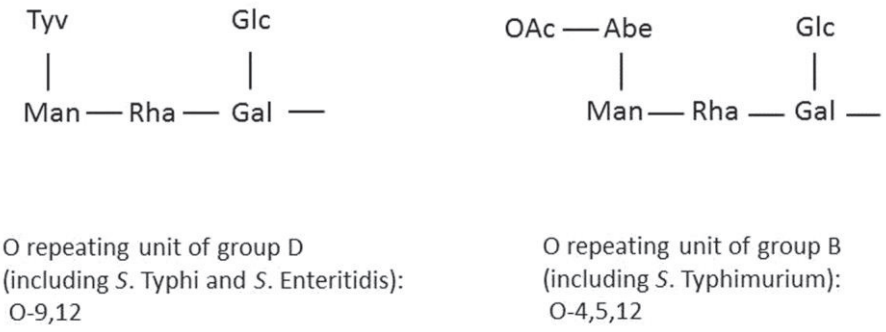

Figure 6. Schematic structure of the O-antigens of *Salmonella* serogroups B and D. Gal=galactose, Glc=glucose, Man=mannose, OAc-Abe=acetylabequose, Rha=rhamnose, Tyv=tylelose. Modified from Saxén.[175]

1.8.5 Shared antigens with *S.* Typhi, *S*. Paratyphi and NTS

Three of the immunologically most relevant antigens of *S.* Typhi are the Vi antigen, H-d antigen and O-antigens O-9 and O-12. Also *S*. Paratyphi A and B express one of these typhoidal O-antigens O-12. *S*. Paratyphi C has no common O-antigens with *S*. Typhi, but it carries the Vi antigen. Many NTS serotypes share one or two of the typhoidal O-antigens with *S*. Typhi (see Table 1). Such NTS serotypes include the two most common isolates[129, 156, 169, 170] of human non-typhoid salmonellosis: *S*. Enteritidis carries both of the typhoidal O-antigens (O-9 and O-12) and *S*. Typhimurium one of these (O-12). Notably, also many other common isolates of iNTS in Africa express the typhoidal O-antigens: *S*. Dublin carries O-9,12 and *S*. Stanleyville O-12.[130, 156, 157] About half of all NTS strains reported to Centers for Disease Control and Prevention (USA) carried one or both of the typhoidal O-antigens: one quarter both O-9 and O-12, and one quarter only O-12.[129]

1.9 Antibody-mediated immunology against Salmonella

1.9.1 Immunity against *Salmonella*

The host defense mechanisms against *Salmonellae* include the natural barrier functions of the intestine such as intestinal microbiota, the mechanisms of innate immunity, and adaptive immunity, which comprises both local secretory antibodies and systemic antibodies as well as cell-mediated immune mechanisms.[34, 172, 176] This thesis focuses only on the antibody-mediated response.

An effective mucosal immune response against *Salmonellae* prevents the first stage of the infection by inhibiting colonization in the gut mucosa; it blocks the invasion of the pathogen into epithelial cells and prevents bacteria from reaching the lamina propria, thereby inhibiting the subsequent stages of the infection process.[25]

1.9.2 Antibody-mediated immune response elicited in typhoid fever

S. Typhi infection elicits antibodies against O-, Vi, and H-antigens, and various protein antigens. These antibodies can be found in the serum[177, 178] and in intestinal secretions.[177, 179] However, the immune response elicited in typhoid fever does not always result in protection, but, instead, relapses and re-infections may occur in 15–20% of individuals recovering from a clinical infection.[25] Bhaskaram et al. reported that a minority of patients with typhoid fever had no detectable levels of serum antibodies to *S*. Typhi.[33] Dupont et al. have reported that an earlier *S*. Typhi infection protects poorly in a re-challenge with 100 000 virulent *S*. Typhi; volunteers with prior typhoid fever had re-infection in 23% of cases and volunteers without prior infection became infected in 30% of cases in experimental challenge studies with 22 volunteers who had one year before recovered from induced typhoid fever and 34 controls.[180]

1.9.3 Antibodies against O-antigen of *Salmonella*

O-antigens cover most of the surface area of *Salmonella;* therefore, it is not surprising that our immune system produces antibodies against them. In the intestine of mice, Sal4 antibodies against the O-antigen of *Salmonella* have been shown to prevent the earliest steps of infection by blocking the invasion of *Salmonella* into epithelial cells in the absence of other immune or non-immune protective mechanisms.[181] It promotes bacterial agglutination and clearance via immune exclusion,[181] inhibits the flagellum-based motility of the *Salmonella,*[182] and has immediate effects on the activity of the type three secretion system, membrane energetics, and outher membrane integrity.[183] By at least these mechanisms, it renders *Salmonella* temporarily avirulent.[183]

Human serum IgG and IgM antibodies against O-antigen have also been demonstrated to bring about complement-mediated killing of *Salmonella*.[184, 185] The same has been shown earlier in animal experiments.[186] Systemic O-antigen-specific IgM antibodies have proved more protective than the corresponding IgG antibodies after intraperitoneal *S. Typhimurium* challenge in mice.[175, 187, 188]

1.9.4 Protective capacity of O-antigen-specific antibodies against NTS

NTS O-antigen-specific antibodies have been reported to carry protective capacity against salmonellosis in studies in humans[184] and experimental animals.[181, 186-193]

MacLennan et al. have shown a protective role for antibody-induced complement-mediated killing of NTS in African children with serum antibodies to O-antigen.[184] They found a relative absence of NTS bacteremia among children aged 1–4 months and a peak in NTS bacteremia after the maternal antibody levels have waned[194] and the infant's own antibody production against NTS has not yet developed sufficiently to provide protection.[184] They have also shown, in Africans, an increase in anti-*Salmonella* IgG and IgM antibody titers, and bactericidal activity of serum against NTS with age, corresponding to a decline in NTS bacteremia cases.[184] Collectively, these observations suggest that serum *Salmonella*-specific antibodies may serve to protect against iNTS. Interestingly, in patients infected with HIV, an overproduction of *S.* Typhimurium O-antigen-specific IgG has been reported, and in contrast to studies in healthy children, these antibodies have been found to correlate with impaired immunity against iNTS.[195] This dysregulation in HIV-infected persons has only been associated with antibodies of the IgG isotype.[195] Trebicka et al. have recently demonstrated that O-antigen-specific antibodies can also inhibit killing of *Salmonella* in healthy adults. However, this result was shown only with serum of one individual.[185] In contrast to this, all other serum samples of healthy Americans (49/50) in their study were found to promote killing of *Salmonella* in a O-antigen-specific antibody-mediated complement-dependent way.[185] This bactericidal activity increased an age-dependently.[185]

In animal studies, intestinal or mucosal S-IgA against *S.* Typhimurium O-antigens have proved protective,[181, 189-192, 196] even in the absence of other immune mechanisms.[181] Michetti et al. have shown with mice that a monoclonal, polymeric IgA antibody against the *Salmonella* O-antigen (Sal4) transported into the intestinal tracts was sufficient to protect the animals against an otherwise lethal oral dose of virulent *S.* Typhimurium.[181, 190] In addition to this, Endt et al. have demonstrated in mice that immunization with *S.* Typhimurium elicits an adaptive immune response, conferring protection against mucosal disease on re-infection with *S*. Typhimurium. This protection proved to be O-antigen-dependent.[191] Saxén et al. have shown that systemic O-antigen-specific IgM antibodies are more

protective than IgG antibodies against intraperitoneal *S. Typhimurium* challenge in mice. After intravenous challenge, however, these antibodies did not prove protective.[175, 175, 186-188]

1.10 Vaccines against typhoid fever

The first typhoid vaccines, introduced as early as in 1896,[197] were the parenteral whole-cell preparations inactivated with various methods. The efficacy of acetone-inactivated vaccine was shown to be 80% (61% to 90%), formol-inactivated 77% (60% to 87%), heat-inactivated 73% (61% to 82%), and alcohol-inactivated 58% (34% to 73%).[198] Despite the considerable efficacy of most of these preparates, these vaccines are no longer available due to the high rate of adverse effects that they caused (9-34%).[198] The reactions were considered to be caused by the lipopolysaccharide part of the bacteria acting as an endotoxin.[199]

Three vaccines are currently available for clinical use in the prevention of typhoid fever: the oral live attenuated *Salmonella* Typhi Ty21a vaccine (Ty21a vaccine) and the parenteral Vi capsular polysaccharide vaccine (Vi vaccine), and the Vi conjugated vaccine (Vi tetanus toxoid, PedaTyph, Vi).[200] The third vaccine is licensed only in India for local use with limited data on safety and immunogenicity.[201-203] Both Ty21a and Vi vaccines have proven immunogenic, providing similar levels of protection against typhoid fever in clinical trials.[150, 198, 204]

1.10.1 *Salmonella* Typhi Ty21a vaccine

The oral *Salmonella* Typhi Ty21a vaccine (Vivotif®) is a live, attenuated whole-cell preparation containing all structures of the *S*. Typhi other than the Vi antigen. The Ty21a strain was developed in the 1970s by mutagenesis of parental strain *S*. Typhi Ty2 by generating *gal* E mutants, which have the enzyme (UDP)-galactose-4-epimerase fully blocked.[205] This enzyme is essential when UDP-galactose is transformed to UDP-glucose, and therefore the *gal* E mutants cannot synthesize the complete LPS core and O-9,12 antigens in the absence of galactose. In the gut, galactose is available and LPS O-9,12 antigens are synthetized to ensure proper immunogenicity. In the gut, galactose is thus accumulated in *gal* E mutant bacteria in the form of galactose-1-phosphate and UDP-galactose which initiate the

bacteriolysis and destroy the vaccine strain.[205] In addition to the non-functional UDP-galactose-epimerase gene, the vaccine strain cannot at least express the Vi antigen[150] and it cannot cause disease.[206]

The Ty21a vaccine has been reported to confer 51–69% protection against *S*. Typhi in field trials using the vaccine as enteric-coated capsules.[123, 207, 208] The same vaccine formulation proved to offer a 62% protection over seven years. Three doses of liquid formulation (every other day schedule) elicited 77% protection over three years and 78% over five years.[207] This vaccine was found to induce herd immunity in endemic areas.[123, 204, 208]

Nowadays, the Ty21a vaccine is available solely as enteric-coated capsules. It is recommended to be used only for those over 2-6 years of age, the recommendations varying between various countries. The vaccination schedule comprises three (Europe) or four (USA and Canada) capsules, which are administered two days apart.[25, 138, 150, 203] In Europe, a three-dose booster is recommended every three years for people living in endemic areas. For those travelling from non-endemic to endemic areas booster immunization are recommended every 1-7 year depending on national policies.[138]

The protective immune mechanisms induced by the Ty21a vaccine include both humoral and cell-mediated immune mechanisms.[23-32] This thesis focuses only on the antibody-mediated response. The Ty21a vaccine elicits both mucosal and serum antibody responses. The gut-derived O-antigen-specific antibody-secreting cells in the circulation and serum IgG have been suggested as surrogate markers for protection.[25, 150, 209] The numbers of plasmablasts increase with increasing number of Ty21a vaccine doses.[23] This occurs analogously with the increase in protective efficacy against typhoid fever shown in field trials.[123]

1.10.2 Vi capsular polysaccharide vaccine

The Vi antigen is one of the major virulence factors of *S.* Typhi.[210] The Vi capsular polysaccharide vaccines (Typherix® and Typhim®) have been purified by a non-denatured purification method from strain *S.* Typhi Ty2. These currently available parenteral vaccines cause only a low rate of adverse effects.[199] However, they

confer a protection of 55–72% against typhoid fever,[204, 211-214] providing also herd immunity.[214]

The Vi vaccine is given intramuscularly. It should not be given to children under two years of age as this population is unable to mount adequate immune responses to pure polysaccharide vaccines, such as the Vi vaccine. A booster dose is recommended at two to three-year intervals.[203]

The Vi vaccine contains purified Vi polysaccharide. It confers protection by T cell-independent antibody production against Vi antigen.[150] It does not induce an immunological memory: revaccination does not offer a boosting effect.[215] The Vi vaccine elicits seroconversion in 75%-95% of vaccinees.[199, 212, 215, 216] The protective level of serum antibodies is obtained within one week and maximum concentration in 28 days after immunization.[150, 216] Serum antibodies persist in adults for at least three years.[217] The Vi vaccine does not induce mucosal immunity.[25, 150, 215]

1.10.3 Cross-protection against paratyphoid fever by the Ty21a, TAB or Vi vaccine

No prospective field trials exist on the cross-protective potential of typhoid vaccines. A retrospective study exploring imported cases of enteric fever in Israeli travellers to India showed that the Ty21a vaccine may confer some cross-protection against *S.* Paratyphi A.[142] In Israel, travellers were immunized with the Ty21a vaccine until 2001 and after that with the Vi vaccine. The general attack rate by *S.* Paratyphi A was 0.26 in 10 000 until 2001, i.e. at the time Ty21a was used, and 0.79 in 10 000 when the Vi vaccine was given.[142]

Recently, Levine et al. revisited old data from randomized controlled field trials on typhoid vaccination in Chile with 49 790 volunteers vaccinated with Ty21a and 49 211 volunteers receiving placebo. In that study, the protection rate against typhoid fever was 58%, yet the new analysis revealed a simultaneous cross-protective rate of 49% against *S*. Parayphi B.[22] This cross-protection was thought to be mediated by common O-antigen (O-12) with *S*. Typhi and *S*. Paratyphi B.[22] In that study, the number of cases with *S*. Paratyphi A proved too low to allow an evaluation of cross-protective efficacy against *S*. Paratyphi A infection.[22]

In contrast to the studies described above, in a large field trial in Plaju, Indonesia, with 10 275 volunteers immunized with the Ty21a with liquid formula or with enteric-coated capsules (both with 3 doses at one-week intervals), protection was not afforded against *S*. paratyphi A.[218] The protective efficacy against typhoid fever was only 53% with the liquid formula and 42% with capsules.[218] One plausible explanation for the low protective rate against *S*. Typhi and the lack of cross-protection against *S*. Paratyphi A is in the vaccination schedule; when vaccine doses are given one week apart, as opposed to using the recommended two-day interval, the first dose(s) manages to stimulate an intestinal immune mechanism, which acts against the subsequent doses of this vaccine (discussed more detailed in Section 1.11.5). The actual number of doses given in Plaju thus corresponds to only one dose.

There are two retrospective studies evaluating the protective efficacy of the old parenteral whole-cell vaccine TAB against *S.* Paratyphi A. In a study with travellers to Nepal, the majority of whom had been immunized with TAB and some with the Ty21a vaccine, an overall efficacy of 72-75% was found against *S.* Paratyphi A and 95% against *S*. Typhi.[219] By contrast, after introduction of the parenteral TAB vaccine in the national vaccination program in Thailand a decrease in *S.* Typhi- but not *S.* Paratyphi A isolates during a typhoid fever epidemic was observed.[220]

The Vi vaccine has been reported to offer no protection against paratyphoid fever in a field trial with 65 287 volunteers in China.[213] In the retrospective study by Meltzer et al. described above, the general attack rate by *S*. Paratyphi A increased when the Vi vaccine was used instead of the Ty21a vaccine.[142, 213] Likewise, an increase in the number of paratyphoid A cases was reported after the introduction of the Vi vaccine in Guangxi, China.[221]

1.10.4 Cross-reactivity to paratyphoid fever elicited by the Ty21a, TAB or Vi vaccine

Some previous immunological studies have shown immunological cell-mediated cross-reactivity against paratyphoid serotypes in volunteers immunized with the Ty21a vaccine. Tagliabue et al. demonstrated in Ty21a-immunized volunteers cross-reactive IgA antibody-dependent cell-mediated responses against *S*.

Paratyphi A and B but not C.[16] Post-immunization serum samples from Ty21a-recipients and mononuclear cells were able to kill *S.* Typhi, *S.* Paratyphi A and B, but not *S.* Paratyphi C or *Salmonella Tel Aviv*. The first two serotypes share O-12 antigen with *S*. Typhi, while the latter two lack O-antigens in common with *S*. Typhi. Later, D'Amelio[18] and Nishini[17] et al. reported similar responses to paratyphoidal serotypes with a cell-mediated antibacterial assay. Recently, Wahid et al. demonstrated that Ty21a elicits an O-antigen-specific cross-reactive response against *S*. Paratyphi A and B.[19] They also showed that opsonization with post-vaccination sera predominantly increased phagocytosis of these bacteria. This activity correlated with levels of O-antigen-specific antibodies. [21]

To my knowledge, prior to this thesis, no previous studies have been conducted on the cross-reactive immune response against *S*. Paratyphi elicited by the Vi vaccine.

1.10.5 Cross-reactive immune response to NTS elicited by the Ty21a or Vi vaccine

Previously, in preliminary experiments setting up the ELISPOT assay in our lab for the first time, the Ty21a vaccine was shown to elicit a response of circulating plasmablasts cross-reactive with *S*. Typhimurium SL3261 sharing O-12 with *S*. Typhi[63] and with *S*. Typhimurium SL2404, which is a derivative of *S*. Typhimurium SL3261 with rfb of *S*. Enteritidis that expresses both typhoidal O-antigens (O-9 and O-12).[20, 23, 62, 63] In addition to the cross-reactive plasmablast responses, serum antibody response cross-reactive with *S*. Typhimurium SL2404 has been described.[23]

The Vi vaccine has been shown to elicit serum antibodies against *S*. Enteritidis (sharing O-9 and O-12 antigens with *S*. Typhi) with 26% or 83% seroconversion depending on the LPS content of the vaccine (0.2% and 5%, respectively).[199]

1.11 Plasmablast response to S. Typhi elicited by the Ty21a vaccine

S. Typhi-specific plasmablasts, identified as specific antibody-secreting cells (ASCs) have been demonstrated to appear in the peripheral blood after immunization

with the *Salmonella* Typhi Ty21a vaccine administered either orally as a live attenuated[20, 23, 222] or a killed whole-cell[62] vaccine or as a killed parenteral whole-cell[62] or live rectal vaccine.[119]

1.11.1 Kinetics of the ASC response

Kinetics of the ASC response was similar regardless of whether an oral, parenteral, or rectal vaccine formula was used.[20, 23, 62, 119] Antigen-specific ASCs are known to appear in the peripheral blood 2-4 days after antigen encounter in humans [20, 23, 62, 119] The peak in the magnitude of the ASCs is seen around day 5 for IgM-ASC and on day 7 for IgA- and IgG-ASC. Likewise, the sum of IgA-, IgG-, and IgM-ASC is highest on day 7. Antigen-specific ASCs mostly disappear from the blood by day 14-16 after immunization.[23, 57, 62, 119] Notably, when the exposure of an antigen is prolonged, the time period when specific ASCs can be found in the blood is longer, with no distinct peak; instead, the response stays high over several days.[56, 69] This was initially shown by comparing the regular dosing schedule of three doses of oral Ty21a vaccine given two days apart with a prolonged schedule of six doses with the same interval.[69] Later, these kinetics were confirmed in patients with bacterial gastroenteritis; ASCs were found in the circulation as long as the pathogen persisted in the intestine.[56] The kinetics of the response after booster immunization differs from that described above for primary immunization; the booster response has been reported to peak already on day 5 and in most of the cases to fade away by day 9 post-vaccination. Later, similar kinetics of booster immunization with the oral cholera vaccine has been reported.[223, 224]

1.11.2 Ig-class distribution of ASC response

The Ig-class distributions of ASC response elicited by live or killed oral, live rectal, and killed parenteral vaccines are different. After immunization with live oral, killed oral,[62] or live rectal vaccine[119, 225] the responses are dominated by IgA in most of the vaccinees and IgM dominates more often than IgG. After immunization with killed parenteral vaccine, the plasmablast response was dominated by IgM in most of the recipients.[62]

1.11.3 Specificity of response

The specificity of the ASC response was different after immunization with the oral and parenteral Ty21a vaccines. After oral immunization, the ASC response was directed mostly to the O-antigens, while after parenteral immunization, an equal response to the O-antigens, the LPS core, and the flagella (H-antigen) has been reported.[62]

1.11.4 Homing profile of ASC response

Primary immunizations with the oral or rectal Ty21a vaccine have been found to elicit a gut-directed ASC response with all cells (99%) expressing $\alpha_4\beta_7$, the intestinal HR, and a smaller proportion (42%) L-selectin, considered to be associated with more systemic homing.[80, 119, 225] After parenteral immunization, by contrast, a more systemic homing profile has been reported; most of the cells (86%) express L-selectin and a smaller proportion (58%) $\alpha_4\beta_7$.[80] After booster immunization with the oral Ty21a vaccine, high proportions of both $\alpha_4\beta_7$ and L-selectin-expressing cells have been seen (90% and 88%, respectively).[226] In contrast to this, after a parenteral booster immunization with Ty21a, the HR profile has been found to be similar to that observed after primary immunization with the parenteral vaccine.[226] Similarly, the homing profile of the response to rectal booster immunization of volunteers primed orally by Ty21a has proven similar to that after primary immunization (95% or 99% of ASCs expressed $\alpha_4\beta_7$ after primary or booster immunization, and 36% or 38% expressed L-selectin, respectively).[225]

1.11.5 Magnitude of ASC response and vaccine efficacy

The magnitude of the specific plasmablast response parallels the results obtained for protection in field trials.[118]

The magnitude of the plasmablast response was reported to depend on the following factors: 1. the nature of antigen (killed vs. live), 2. the number of doses administered, 3. the vaccine formula, and 4. the interval between the doses.

The oral live vaccine elicited a significantly higher plasmablast response than the oral killed or the killed parenteral vaccine.[62] This was suggested to be due to the ability of the living bacteria to multiply and colonize the mucosa.[118, 227]

The number of plasmablasts increases with increasing number of doses.[23] A single dose of the Ty21a vaccine has been shown either to induce no plasmablast response or to elicit a response of low magnitude.[23] Two doses elicit a higher response than one dose, and the greatest response is seen after administration of three doses.[23] As described above, administration of six doses does not elicit a higher peak of the plasmablast response, but instead, the response remains high for a longer period than after immunization with three or four doses.[69, 119] Furthermore, it has been speculated that six doses elicits a more effective immune response than three because the total number of the plasmablasts recruited is higher.[118] The increasing magnitude of the plasmablast response with increasing numbers of doses has been shown with Ty21a in enteric-coated capsules [23] and is in line with increasing efficacy in field trials with the same regimen.[123, 124]

The vaccine formulation has an effect on the magnitude of the plasmablast response as well as on the vaccine efficacy.[118] Kantele et al. compared the responses after three different orally administrated vaccine formulas (gelatin capsules, enteric-coated capsules, and suspension formulation) with equal doses and dosing schedules[23] The lowest response was elicited when the vaccine was administered in gelatin capsules, and the highest with the suspension formulated regimen.[23, 63] In line with the magnitude of the ASC responses, suspension and enteric-coated capsule formulas were found to have a significantly better protective efficacy than gelatin capsules in a field trial in Santiago.[125] Furthermore, in another trial in the same region the suspension formulated vaccine was superior in efficacy over the enteric-coated capsules,[228] consistent with the differences in magnitude of the ASC responses.[23]

The interval between the doses influences the response significantly. When booster immunization with live oral Ty21a vaccine was performed soon after previous vaccination (2-6 months), an absent or low ASC response was elicited,[229, 230] in contrast to immunization with a longer interval between the primary and secondary dose (1-2 years).[231] In the latter situation, the ASC response was higher after booster immunization than after primary immunization. [231] The ASC response

was higher after booster immunization than after primary immunization.[231] The low plasmablast response detected after a short vaccination interval is presumably due to active intestinal immunity elicited by the initial doses; vaccine-elicited antibodies can inhibit the multiplication, adherence, or invasion of the bacteria of the live booster dose.[229, 230] Therefore, active mucosal immunity reduces the effective dose of the second vaccine. Active immunity is reached within one or two weeks after immunization with the Ty21a. Notably, this could also explain the low protective efficacy found in the field trial by Simanjuntak et al.[218] They administered the Ty21a vaccine doses seven days apart and found only 42% protective efficacy against typhoid fever with three enteric-coated capsules and 53% with the liquid formula.[218] Respective results of other studies in other locations using a two-day interval schedule, the protective efficacy has been 67%[232] and 96%.[233]

2 AIMS OF THE STUDY

A series of studies explored the specific and cross-reactive humoral immune responses in volunteers immunized with the *Salmonella* Typhi Ty21a or the parenteral Vi capsular polysaccharide vaccine or in patients with natural enteric fever. The more specific aims were as follows:

1. To evaluate the distribution of *S*. Typhi-specific and total IgA1 and IgA2 subclasses between various secretions and *S*. Typhi-specific and total circulating IgA1- and IgA2-plasmablasts and their homing profiles after oral vs parenteral *Salmonella* Typhi vaccinations (Study I).

2. To compare head-to-head the typhoid-specific plasmablast and antibody responses between volunteers vaccinated with the oral *S*. Typhi Ty21a or the parenteral Vi capsular polysaccharide vaccine (Study IV).

3. To identify cross-reactive antibody-mediated responses to *S*. Paratyphi and NTS as elicited after immunization with the Ty21a vaccine (Studies II and III), the Vi vaccine (Studies V and VI) and in natural typhoid or paratyphoid fever (Studies II and III).

4. To compare head-to-head the cross-reactive antibody-mediated responses to *S*. Paratyphi and NTS between Ty21a- and Vi-vaccinated age- and gender-matched volunteers (Studies V and VI).

3 MATERIALS AND METHODS

3.1 Study design

The antibody-mediated immune responses were examined at a single-cell level in volunteers immunized with different typhoid fever vaccines and in patients with enteric fever. The investigated vaccines were the attenuated oral whole-cell *Salmonella* Typhi Ty21a vaccine (in all studies), rectal and parenteral formulas of the Ty21a vaccine (not commercial) (in Study I) and the Vi capsular polysaccharide vaccine (Studies III-VI).

In this thesis, circulating plasmablasts specific to *S.* Typhi (Studies I-VI), to *S*. Paratyphi A/B/C (Studies II and V), and to different NTS serotypes (Studies III and VI) were evaluated. *Yersinia enterocolitica* was used as a negative control (in Studies III and VI). Specific plasmablasts were identified by enzyme-linked immunospot assay (ELISPOT) as antigen-specific antibody-secreting cells (ASCs) (IgA, IgG, and IgM in Studies I-VI and IgA1 and IgA2 in Study I). In a subgroup of vaccinees, the homing potentials of plasmablasts were characterized by combining immunomagnetic cell sorting with the ELISPOT (Studies I-IV and VI). Levels of specific antibodies (IgA, IgG, and IgM) were determined by ELISA in serum and ALS (antibodies in lymphocyte supernatants) samples (Studies III, IV, and VI), and the IgA1 and IgA2 ratio was determined in serum and various secretions after the immunizations (Study I).

3.2 Ethics statement

The study protocol was approved by the ethics committee of Helsinki University Central Hospital (Dnr/382/E7/07 and Dnr/411/E5/02) and the Finnish Medicines Agency and registered in the Current Controlled Trials Ltd. c/o BioMed Central (ISRCTN68125331). Written informed consent was obtained from all study subjects.

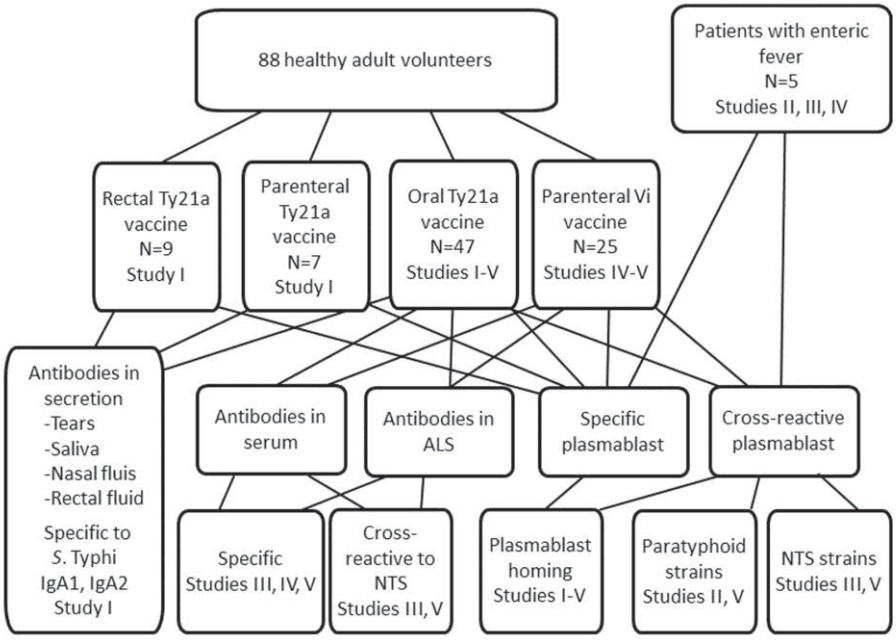

Figure 7. Study protocol.

3.3 Volunteers

A total of 88 healthy volunteers (aged 18-62 years, median 26) with no history of enteric fever or typhoid vaccinations and no other immunizations received within the last four weeks were enrolled in the vaccination studies (Studies I-VI).

Five patients with enteric fever were also enrolled to allow a comparison between responses to vaccination and to natural *S*. Typhi or *S*. Paratyphi A infections (Studies II-IV). The typhoid fever patients (three females, one male; aged 22–30 years) included two Finnish-born travellers returning from Central-America and India, a Sri Lankan applying for asylum in Finland, and a Nepalese immigrant with an infection relapse one month after the first episode. The patient with paratyphoid fever was a 30-year-old female immigrant from India. All of these patients were treated at the Division of Infectious Diseases, Helsinki University Hospital. Typhoid and paratyphoid fever were diagnosed by blood culture. *S*.

Typhi strains of the patients were all Vi antigen positive. Paratyphoid fever was caused by *S*. Paratyphi A strain.

3.4 Vaccinations

Volunteers were vaccinated with one of the following vaccines: 1) the live attenuated oral vaccine containing ≥2x10^9 live *Salmonella* Typhi Ty21a bacteria (Vivotif®, Crucell NV, Leiden, the Netherlands) (in all studies); 2) the live Ty21a vaccine suspended in 0.9% NaCl and administered intrarectally (Study I); 3) a parenteral typhoid vaccine containing at least 0.5x10^9 formalin-killed whole bacteria/dose prepared from strain Ty21a in Swiss Serum and Vaccine Institute (currently Crucell NV) (Study I); 4) the parenteral Vi capsular polysaccharide vaccine (Typherix®, GlaxoSmithKline Biologicals s.a., Rixensart, Belgium) (Studies IV-VI).

The oral vaccine was administered as one capsule daily on three (Studies II-VI) or four (Study I) days, one day apart. The rectally administered Ty21a was given as four doses at one-day intervals. The rectally administered Ty21a was obtained from the enteric-coated capsule by opening the capsule and suspending its content in 1 ml of sterile saline. This suspension was administered in the rectum with a 10-cm-long 1-ml syringe. The killed whole-cell parenteral Ty21a was prepared according to a previously established protocol.[62] It was administered as one 0.5-ml dose intramuscularly on day 0 in the left arm of the volunteer.

In age- and gender-matched studies (IV-VI), the vaccine type was given randomly to the first volunteer of each age and the other vaccine type was given to an age- and gender-matched subject.

3.5 Samples

Blood samples

On the basis of the previously characterized[23] kinetics of lymphocyte circulation, blood samples for plasmablast assays were drawn in lithium-heparin tubes before and 7 days after the vaccination or, for patients with enteric fever, 7–10 days after

the onset of the infection symptoms. The samples were processed within one hour. Serum samples were collected before and 28 days after vaccination.

Samples of the external secretions

Twelve female volunteers who had been immunized orally (n=7) or rectally (n=5) with the Ty21a vaccine provided samples of external secretions (tears, parotid saliva, nasal wash, intestinal lavage) before and 28 days after immunization for analysis of total and *S.* Typhi-specific IgA1 and IgA2 antibody levels. All samples were collected during one day. All volunteers had regular menstrual cycles; two were on birth control pills.

Tears were collected with a capillary tube from the medial corner of the eye after stimulation with splatter of an orange peel. Nasal wash was obtained by instillation of prewarmed saline in the nostrils and expelling after 30 s. Unstimulated parotid saliva was collected using a Schaefer cup placed over the parotid duct and centrifuged to remove debris. Intestinal secretions were obtained either with rectal wick (Polyfiltronics, Inc., Rockland, MA) or by the oral administration of Golytely (buffered polyethylene glycol solution, Braintree Labs, Braintree, MA), as described previously.[119, 234, 235]

3.6 Antigens for ELISPOT

Altogether 12 different bacterial serotypes and Vi antigen of the vaccine preparation (Typherix®, 10 μg/ml in PBS) were used as antigens in the ELISPOT assays (Table 2). For analysis of cross-reactivity, ten *Salmonella* serotypes that shared at least one O antigenic determinant (9, 12, or both) with *S.* Typhi, and two with different O-antigens (S. Virchow O-6,7; *S*. Hadar O-6,8 and *S*. Egusi O-41) were used (Table 2). *Yersinia enterocolitica* was used as a negative control (Studies III-VI).

The response to *S.* Typhi was measured in Study I using *S.* Typhimurium SL2404 strain (O-9,12) in coated and whole-cell *S.* Typhi in Studies II and III. In Studies IV-VI, the response was calculated as the sum of responses in assays for various typhoidal antigens, the typhoidal O-9,12 antigen (represented by the *S.*

Typhimurium SL2404 strain, which is a derivative of *S*. Typhimurium SL3261 with rfb of *S*. Enteritidis[236]). H-d was represented by the *S.* Egusi and Vi antigen (Typherix vaccine preparation 10 μg/ml in PBS). The latter approach was used to ensure that as many antigenic determinants as possible could be included. To confirm the expression of the O- and Vi antigens, each bacterial strain was analyzed in the Finnish national reference laboratorium (Gastroenterological Unit of the National Institute for Health and Welfare in Helsinki) according to their routines. Bacteria cultured on Luria Bertani plates were suspended in 0.9% NaCl in distilled water, and a sample of each suspension was grown on Drigalski-Conradi plates to determine the concentration of the bacteria in the solution. Immediately after removal of the sample for concentration analyses, the bacteria in the stock solution were formalin-(1%)-killed and stored at -20°C in aliquots until use. Prior to use in the assays, the concentration of this stock suspension was adjusted to 10^9 bacteria/ml in PBS.

Table 2. Bacteria used as coating antigens in the ELISPOT assay, their origin and typhoidal antigens.

		Antigens			
	Strain	**O-9**	**O-12**	**Vi**	**H-d**
***S.* Typhi**	Vsa61	+	+	+	+
Recombinant *S.* Typhimurium[a]	SL2404	+	+	-	-
***S.* Paratyphi A**	RHS6716	-	+	-	-
***S.* Paratyphi B**	RHS6744	-	+	-	-
***S.* Paratyphi C**	ATCC 13428	-	-	+	-
***S.* Egusi (H-d)**	RHS6854	-	-	-	+
***S.* Enteritidis**	RHS634	+	+	-	-
***S.* Typhimurium**	8965	-	+	-	-
***S.* Agona**	RHS6160	-	+	-	-
***S.* Stanley**	RHS6766	-	+	-	-
***S.* Virchow**	RHS6740	-	-	-	-
***S.* Hadar**	RHS148	-	-	-	-
Yersinia enterocolitica	RHI4823	-	-	-	-

[a]Derivative of S. Typhimurium SL3261 with rfb of S. Enteritidis

-*S.* Paratyphi C strain was from the American Type Culture Collection (ATCC, Manassas, VA), while the other strains were from the National Institute for Health and Welfare, Helsinki, Finland.

3.7 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMC were separated using Ficoll-Paque density gradient centrifugation of freshly heparinized venous blood as described previously.[237]

3.8 Separation of receptor-negative and -positive cell populations

The expressions of HR on specific ASC were explored in 34 vaccinees and in all five patients with enteric fever. Separation of the cells into HR-positive and -negative populations has been described earlier.[79, 80] Aliquots of cell suspensions (3.4×10^6 PBMCs were investigated per HR) were incubated for 30 min on ice with monoclonal antibodies against $\alpha_4\beta_7$ (ACT-1, Millennium Pharmaceuticals, Cambridge, MA), L-selectin (Leu 8, Becton Dickinson, Erenbodegem-Aalst, Belgium), or CLA (HECA-452, a gift from Dr. Sirpa Jalkanen, Finland). Next, the cells were washed twice and then incubated on ice for 30 min with Dynal® M-450 magnetic beads coated with sheep anti-mouse IgG (Dynabeads, Dynal Biotech, Oslo, Norway). Receptor-positive and -negative cells were then separated by magnetic separation; cells without beads were separated from cells with beads by applying a magnet outside the test tubes, and the supernatants with receptor-negative cells were collected. The beads were then washed and the magnetic separation was repeated. The receptor-positive cells attached to the beads were suspended in RPMI-1640 medium supplemented with 10% FCS. The efficacy of the cell separation has been previously analyzed with flow cytometer.[80] Separated cells were immediately studied with the ELISPOT assay.

3.9 ELISPOT assay of specific ASC or ISC

The isolated PBMC, and for HR analyses, the receptor-positive and -negative cell populations were assayed for antigen-specific ASCs using ELISPOT as previously described.[23] The principle of the ELISPOT assays has been illustrated in Figure 4. For the antibody-specific ASC assay, 96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with the antigen of interest (shown in Table 2) and for the assay of total ISC with antisera to human IgA or IgM (Dako) or IgG

(Sigma-Aldrich, St. Louis, MO). The plates were incubated for 2 h at 37°C or overnight at 4°C. The plates were washed three times with PBS-0.05%-Tween. Non-specific binding was blocked with 1% BSA-PBS for 2 h at 37°C or overnight at 4°C, followed by three washes with PBS (without Tween). Next, the cells were incubated in the wells in RPMI-1640 medium supplemented with 10% FCS for 2 h at 37°C. Antibodies secreted during this time were detected with alkaline phosphatase-conjugated goat anti-human IgA (1:10000; Sigma-Aldrich), IgG (1:500; Sigma-Aldrich), and IgM (1:5000; SouthernBiotech, Birmingham, England), incubated for 2 h at 37°C or overnight at 4°C. For the IgA-subclass assays, monoclonal antibodies to IgA1 and IgA2 were employed (1:100; Nordic Immunological Laboratories, Tilburg, the Netherlands), incubated overnight at 4°C, followed by incubation of alkaline phosphatase-conjugated anti-mouse IgG (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at 37 °C. The substrate (126 mg/100 ml 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt in 2-amino-2-methyl-1-propanol; both from Sigma-Aldrich, suspended 1:5 in 3% melted agarose) was added to the plates. The spots were enumerated with a microscope (Study I) or with an AID ELISPOT reader (Studies II-VI). Each spot was interpreted as a print of one ASC or ISC, respectively. The specificity, linearity, stability, and intermediate precision of the ELISPOT assay for ASCs were validated before Study II. However, the assay was performed exactly the same before and after validation. A response was defined as at least 3 ASC/10^6 PBMCs and marked as LOD (limit of detection of the response) in the figures. This limit was determined over the assay validation process.

ISC assays were used in Study I to determine total IgA1- and IgA2-immunoglobulin secreting cells and in Studies II-VI as a positive control to detect the viability of cells (assay conditions) and to determine any previously unidentified unknown immunodeficiencies of the volunteer (data not shown).

3.10 ALS cultures

PBMCs were cultured in RPMI-1640 medium supplemented with 3 μg/ml l-glutamine (2 mM), penicillin (100 μg/ml), streptomycin (100 μg/ml), and 10% fetal calf serum in flat-bottomed 96-well plates (Cellstar tissue culture plate, Greiner, Frickenhausen, German) at 37°C in 5% CO_2 (2×10^6 PBMC/200 μl/well).

Supernatants were collected after three days, and stored at −70°C until assayed. The medium and all supplemented materials were obtained from the culture media unit of Haartman Institute, Helsinki, Finland.

3.11 ELISA for serum and ALS samples

Specific antibodies (IgA, IgG, and IgM) in serum and ALS samples were measured with ELISA. Microtiter plates (Polysorp, Nunc) coated with a preparation of LPS isolated from *S.* Typhi, *S*. Enteritidis, or *S*. Typhimurium strains (10 μg/ml, all from Sigma-Aldrich) were incubated overnight at 56°C. The cells were then blocked for 2 h with a 5% milk-PBS solution. Next, the samples were incubated in the wells overnight. Horseradish peroxidase (HRP)-conjugated rabbit anti-human IgA, IgG, and IgM antibodies (IgA and IgG 1:10 000, IgM 1:5000; all from Dako) were used as secondary antibodies, and the color was developed with TMB peroxidase substrate (1:1 3,3',5,5'- tetramethylbenzidine and H_2O_2 in citric acid buffer; KPL, Gaithersburg, MD) and stopped with 0.5M sulfuric acid. A response was defined as at least a twofold increase in the specific antibody titer relative to the prevaccination level.

3.12 ELISA assay for measurement of IgA1 and IgA2 antibodies in saliva, tears, nasal lavage, and rectal wick

Samples of tears, saliva, nasal lavage, and rectal wick were assayed for concentrations of both total IgA1 and IgA2 and *S*. Typhi-specific IgA1 and IgA2 by ELISA as described earlier.[238, 239] The assay of the total IgA1 and IgA2 was performed as follows: briefly, ELISA plates (Maxi-sorb, Nunc) were coated with human Ig-absorbed goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). The plates were then incubated overnight with either mouse anti-human IgA1 (Sigma) or mouse anti-human IgA2 (Recognition Sciences Ltd., Birmingham, UK). Duplicate serial dilutions of sample or standard were incubated overnight. Bound immunoglobulins were detected with peroxidase-conjugated goat anti-human IgA (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Wells were developed with *o*-phenylenediamine and H_2O_2 in citrate-phosphate buffer (pH 5.0). The reaction was stopped with 1 M sulfuric acid after 15 min, and

the absorbance was measured at 490 nm in a V_{max} microplate reader (Molecular Devices Corp., Menlo Park, CA).

The assay for *S.* Typhi-specific IgA1 and IgA2 was performed as described elsewhere.[239] Briefly, a whole-cell preparation of *S.* Typhi Ty21a was used as the coating antigen, the plates were blocked with 5% FBS in PBS-Tween, and serial twofold dilutions of the samples and standards were added. Human sera with known antibody titers against *S.* Typhi Ty21a and LPS were included on each plate as positive controls. Bound antibodies were detected with biotin-labeled mouse monoclonal IgG anti-human IgA1 or IgA2 (Nordic) followed by ExtrAvidin peroxidase conjugate (Sigma-Aldrich). The wells were developed with *o*-phenylenediamine-H_2O_2 substrate (Sigma-Aldrich), and the color reaction was stopped with 1 M sulfuric acid and the absorbance read at 490 nm.

3.13 Statistics

In Study I, the statistical analyses were carried out with Student's t-test (Microsoft Excel), and the results were given as geometric means ± SEM for the number of ASCs and ISCs, and as arithmetic means ± SD for HR expressions.

In Studies II-VI, statistical analyses were carried out with JMP software version 9.0.0 (SAS Institute Inc., Cary, NC). The distributions of the ASCs and HR expressions were tested with Shapiro-Wilk's test. Since not all distributions proved normal even after logarithmic transformations, Bonferroni-corrected Wilcoxon signed-rank test was used for comparisons between various antigens and/or Wilcoxon signed-rank test for comparisons between the two groups (Studies II-IV and VI). When the distributions proved normal after logarithmic transformations, Student's t-test was used for the comparisons (Study V). The results were given as means, medians, and 95% confidence intervals (CI) for the number of ASCs, and as means ± 95% CI for HR expressions. Correlation analyses were performed using the Spearman test.

The proportions of the receptor-positive ASCs were calculated as follows: percentage of receptor-positive cells = (100 x the number of cells in receptor-positive cell population) ÷ (sum of the number of cells in receptor-positive and

receptor-negative cell populations). To obtain reliable statistics for the proportions of cells expressing a given marker, we set an inclusion limit of 20 ASCs that needed to be identified among the cells studied.

Table 3. Baseline characteristics and adverse effects reported in volunteers immunized with the oral, rectal or parenteral Ty21a or with the parenteral Vi vaccine.

	Rectal Ty21a vaccine	Parenteral Ty21a vaccine	Oral Ty21a vaccine	Parenteral Vi vaccine
n	9	7	47	25
mean age (range)	30 (22-36)	27 (24-30)	31 (18-62)	32 (22-62)
female	9	6	32	17
male	—	1	15	8
adverse effects				
fever	—	1	—	1
pain at injection site	—	7	—	2
constipation	—	—	—	1
loose stools	—	—	—	1
stomach ache	—	—	1	—
nausea	—	—	1	—
flatulence	—	—	1	—
tiredness	—	—	1	—

4 RESULTS

4.1 S. Typhi-specific humoral immune response in volunteers immunized with typhoid vaccines

4.1.1 *S.* Typhi-specific ASC responses elicited by the Ty21a vaccine

Before the vaccinations, none of the Ty21a-immunized volunteers had plasmablasts (identified as ASCs) specific to any of the typhoidal antigens, but one volunteer had 5 ASC/10^6 PBMC reactive with whole-cell *S.* Typhi (Figure 9).

Seven days after the vaccinations, circulating *S.* Typhi-specific ASC were seen in all Ty21a-immunized volunteers, whether they had been immunized orally, rectally, or parenterally (Figures 9 and 10), while no response was detected to Vi antigen or H-d (measured only in orally immunized volunteers) (Figure 9). The *S*. Typhi-specific responses were mainly dominated by IgA and IgM in all three Ty21a groups, i.e. in volunteers immunized with the oral, rectal or parenteral Ty21a vaccine (Figure 10). The magnitude of the response was higher after the oral than the parenteral Ty21a vaccine ($p<0.01$). No difference was seen in magnitude after the oral and rectal vaccinations. The isotype distribution of these plasmablast responses in volunteers immunized with the parenteral and rectal Ty21a have not been published previously. Statistical comparisons between the responses against the different antigens in the 25 volunteers immunized with the oral Ty21a vaccine are shown in Table 4 (this group of volunteers had age-and gender matched pairs in the Vi-immunized group).

Table 4. Comparison between plasmablast responses to *S*. Typhi antigens and whole-cell *S.* Typhi. Number of responders and numbers of antigen-specific ASC (ASC/10^6 PBMC) in 25 age-and gender-matched volunteers vaccinated one week earlier with the oral Ty21a or the Vi polysaccharide vaccine (arithmetic means and 95% confidence intervals), and statistical comparison (Wilcoxon signed-rank test without Bonferroni correction between the responses to various antigens and Wilcoxon signed-rank test in comparisons between vaccine groups). The light gray in the background indicates results of the comparisons in Ty21a-vaccinated volunteers; dark gray in Vi-vaccinated volunteers, and white indicates comparisons between the Ty21a *vs.* Vi vaccine groups (Wilcoxon signed rank test). Significant differences are indicated with asterisks (*** p<0.001; ** 0.001<p<0.01; * 0.01<p<0.05). NS=not significant. Measured by [a] *S*. Typhimurium SL2404, [b] *S*. Egusi and [c] the Vi vaccine preparates (10 μg/ml).

Bacterial strain / antigen	Number of responder		Plasmablast response ASC/10^6 PBMC (95%CI)		Comparison of the ASC response light gray: the Ty21a group dark gray: the Vi group white: the Ty21a vs. the Vi group				
	Ty21a	Vi	Ty21a	Vi	O-9,12 antigen[a]	H-d antigen[b]	Vi antigen[c]	Sum of typhoid antigens	*S.* Typhi
O-9,12 antigen[a]	25	22	337 (154-520)	41 (18-64)	***	***	***	***	NS
H-d antigen[b]	1	2	1 (0-1)	1 (0-1)	***	NS	NS	***	***
Vi antigen[c]	0	24	1 (0-1)	107 (51-164)	**	***	***	***	***
Sum of typhoid antigens	25	25	338 (155-521)	149 (81-217)	***	***	***	NS	NS
***S.* Typhi**	25	24	314 (147-481)	101 (50-152)	***	***	NS	***	*

4.1.2 *S*. Typhi-specific ASC responses elicited by the Vi vaccine

Before vaccination, 24 of the 25 vaccinees had no ASC specific to any of the antigens in their circulation (Figure 9). One volunteer had 355 ASC/10^6 PBMC to the whole-cell *S.* Typhi, 40 to O-9,12 and 40 to H-d, but none to the Vi antigen. This volunteer had the flu a one week earlier. It has been previously shown that infections may be associated with polyclonal immune responses.[64-66]

Seven days after the vaccinations, 24 of the 25 volunteers showed a response to the Vi antigen and to whole-cell *S.* Typhi, 22/25 to O-9,12, and 2/25 to H-d (Figure 9). The responses were mainly dominated by IgA and IgM. Statistical comparisons between the responses against the various antigens are shown in Table 4.

4.1.3 Comparisons of *S.* Typhi-specific ASC responses between groups receiving oral Ty21a and the Vi vaccine

Statistical comparisons between the responses in the oral Ty21a and Vi vaccine groups of 25 age-and.gender-matched volunteers are shown in Table 4. The response to the O-9,12 antigen was significantly higher in the Ty21a group than in the Vi group, while the response to the Vi antigen was higher in the Vi group than in the Ty21a group. The total response against *S*. Typhi was evaluated in two different ways: as the response to whole-cell *S*. Typhi and as the calculated sum of antigen-specific responses to the O-9,12, Vi and H-d antigens. The sum of the responses to the various typhoidal antigens (Vi + O-9,12 + H-d) was similar in both vaccine groups. When whole-cell *S*. Typhi was used as antigen, the response was higher in the Ty21a group. The response evaluated by the sum of typhoidal antigens was considered to be the more accurate approach, and therefore, the results of the Vi group were compared to those in Studies V and VI.

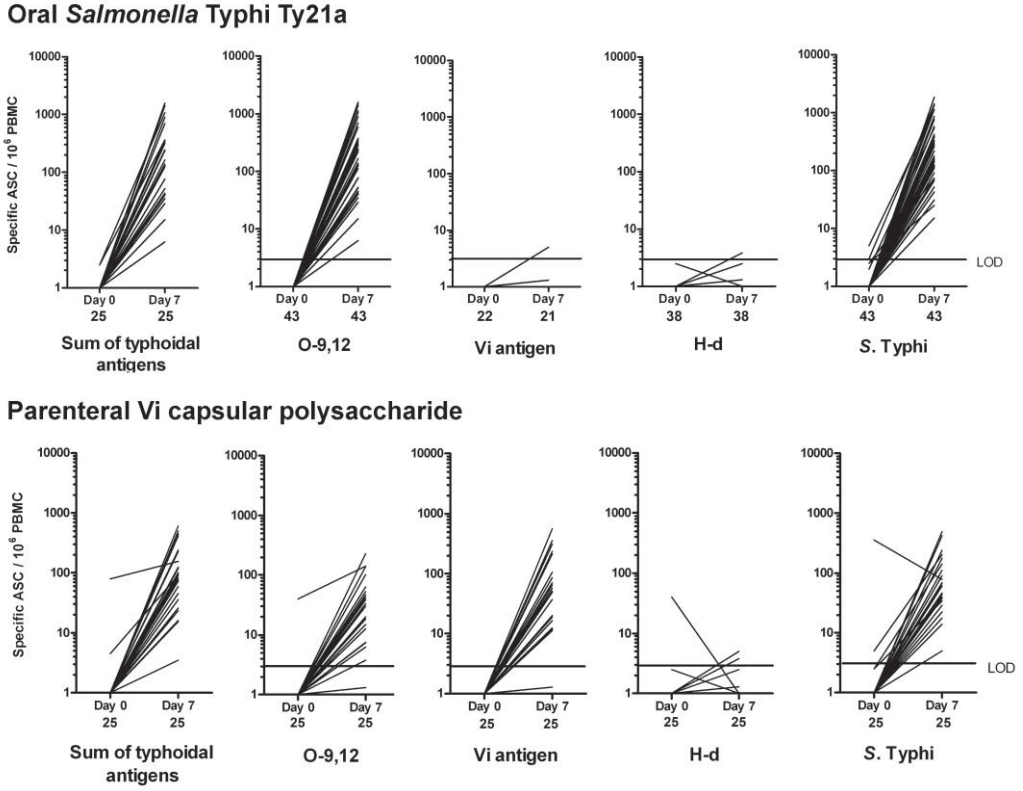

Figure 9. Plasmablast responses specific to various typhoidal antigens and to whole-cell *S*. Typhi before and on 7 after immunization with the oral Ty21a or the parenteral Vi vaccine. Numbers of circulating antigen-specific plasmablasts, identified as antibody-secreting cells (ASC) specific to the typhoidal antigens (O-9,12, Vi and H-d) and whole-cell *S*. Typhi in volunteers immunized with the oral *Salmonella* Typhi Ty21a (three doses) or the parenteral Vi capsular polysaccharide vaccine. The lines represent the sum of Ig(A+G+M) plasmablasts of individual vaccinees on days 0 and 7 after vaccination. The numbers of volunteers for whom the data were pooled are indicated under the data bars. LOD = lower limit of detection of the response. No LOD values are given for Vi + O-9,12 + H-d, since this does not represent a result of the assay, but a calculated total value for three different antigens.

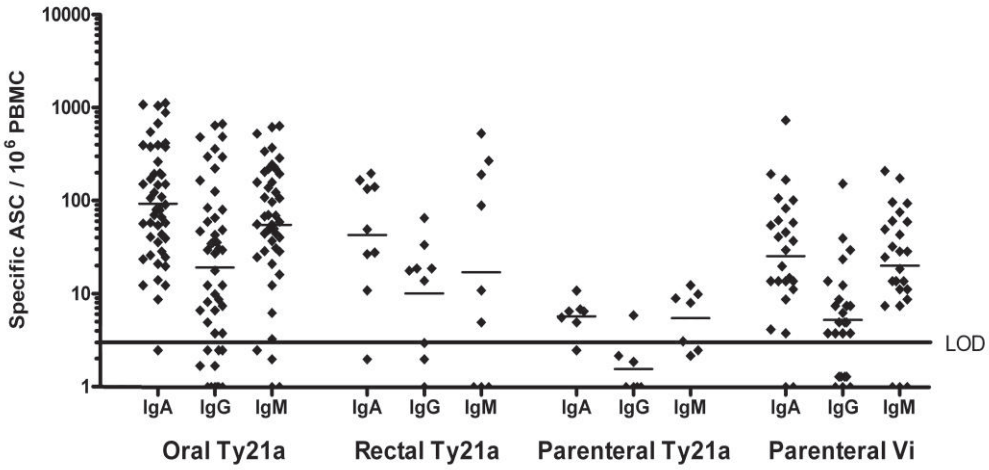

Figure 10. Immunoglobulin isotype distribution of *S*. Typhi-specific plasmablast responses elicited by the oral, rectal, and parenteral forms of the Ty21a vaccine and by the parenteral Vi vaccine. Immunoglobulin isotype distribution of antibodies secreted by plasmablasts, represented as antibody-secreting cells (ASC) reactive with *S*. Typhi (ASC/10^6 PBMC) in volunteers immunized orally (n=47), rectally (n=9), or parenterally (n=7) with a *Salmonella* Typhi Ty21a vaccine or parenterally with the Vi capsular polysaccharide vaccine (n=25). The dots represent results of individual vaccinees and the lines the geometric means of the numbers of ASC-secreting specific antibodies of the IgA, IgG or IgM isotype on day 7 after vaccination.

4.1.4 *S*. Typhi-specific immune responses in assays of the ALS and serum samples and ELISPOT

The *S*. Typhi-specific immune responses elicited by the oral Ty21a and Vi vaccine among 25 age-and gender-matched volunteers were measured with three different assays: by ELISPOT (results shown above) and by measuring antibodies in supernatant of plasmablast culture (ALS) and in serum by ELISA. The percentages of vaccinees with a twofold or higher rise in antibody titers specific to *S*. Typhi in serum and ALS samples and vaccinees responding in ELISPOT are shown in Table 6. ALS responses were found in 92% and 9% of vaccinees in the Ty21a and Vi groups, respectively, while serum antibody responses were seen in 52% and 32% of vaccinees in the Ty21a and the Vi groups, respectively. Percentages of the immunized volunteers responding in serum and ALS samples were smaller than of those responding in the ELISPOT assay (Table 5).

Correlation analyses between the results obtained in the different assays were performed in the Ty21a group in Study III. A correlation was found between the IgA responses in the plasmablast assays, ALS and ELISPOT ($r=0.942$ $p<0.001$). A correlation was found between the IgM responses ($r=0.625$, $p<0.001$) and between the IgG responses. Responses in the plasmablast assays showed no correlation with serum antibody assays in any of the three investigated Ig isotypes.

Table 5. *S*. Typhi-specific immune responses to Ty21a and Vi vaccine in the assays of serum antibodies, ALS and ELISPOT. Percentages of vaccinees responding in serum, ALS culture and ELISPOT assays for *S.* Typhi in 25 age-and gender-matched volunteers immunized with the oral Ty21a or the Vi polysaccharide vaccine. In the ELISA assays (serum and ALS), a responder was defined as an individual with at least a twofold increase in titer (in IgA, IgG, and/or IgM isotype). In the ELISPOT assay, a responder was defined as having at least 3 ASC/10^6 PBMC. Blood samples for ELISPOT and ALS analyses were collected on days 0 and 7, and serum samples on days 0 and 28 after vaccination. The number of vaccinees tested with each assay is indicated in the table. The ELISA assay was only based on *S*. Typhi LPS, not on the Vi antigen.

Assay	Ty21a vaccine (%)				Vi vaccine (%)			
	IgA	IgG	IgM	IgA/IgG/IgM	IgA	IgG	IgM	IgA/IgG/IgM
Serum antibodies ELISA Ty21a: n=25, Vi: n=25	28	20	48	52	16	16	16	32
ALS ELISA Ty21a: n=12, Vi: n=11	92	33	83	92	9	0	9	9
ELISPOT Ty21a: n=25, Vi: n=25	96	72	96	100	88	76	88	96

4.1.5 Distribution of *S.* Typhi-specific IgA1 and IgA2 in various body fluids and *S.* Typhi-specific IgA1 and IgA2-ASC in peripheral blood

The total and *S.* Typhi-specific IgA1 and IgA2 subclass distributions were determined in the various body fluids collected from 12 volunteers immunized with the oral or rectal Ty21a vaccine (Table 6). As to the distributions of the total concentrations of IgA1 and IgA2 in the various secretions, IgA1 was more abundant than IgA2. The rectal samples proved to be the only secretion in which the total concentration of IgA2 exceeded that of IgA1 (Table 6). *S.* Typhi-specific IgA1 and IgA2 were found in all secretions. The concentrations of *S.* Typhi-specific IgA1 were higher than specific IgA2 in all secretions (Table 6). This is consistent with the findings of IgA subclass distribution of circulating *S.* Typhi-specific IgA1- and IgA2-ASC in the peripheral blood, as shown in Table 7. While the secretions were only collected in the orally and rectally immunized volunteers, the dominance of IgA1-plasmablasts was seen also in the parenterally immunized group.

Table 6. *S.* Typhi-specific and total IgA1/IgA2 ratio in secretions after immunization with the Ty21a vaccine. *S.* Typhi-specific and total IgA1/IgA2 ratio in saliva, nasal lavage, tears and elution from rectal wick 28 days after immunization with the oral (n=7) or rectal (n=5) Ty21a vaccine (arithmetic mean ± SD). Data of orally and rectally immunized volunteers are pooled together (n=12).

Secretion	IgA1/IgA2 ratio	
	S. Typhi-specific	Total
Saliva	5.3±3.9	1.9±3.0
Nasal lavage	6.8±4.6	2.1±1.2
Tears	3.9±2.8	1.1±0.4
Rectal wick	2.9±1.1	0.4±0.2

Table 7. *S.* Typhi-specific IgA1-and IgA2-ASC after immunization with the Ty21a vaccine. Numbers of *S.* Typhi-specific IgA1- and IgA2-secreting plasmablast/10^6 PBMC in the peripheral blood of volunteers immunized with a Ty21a vaccine orally, rectally or parenterally (geometric mean ± SEM; %IgA1 given as arithmetic mean)

	Oral	Rectal	Parenteral
IgA1-ASC	69 ± 40	21 ± 20	15 ± 3
IgA2-ASC	18 ± 27	8 ± 5	8 ± 3
% IgA1	74 %	76 %	64 %
n	12	11	7

4.2 Cross-reactive humoral immune responses in volunteers immunized with typhoid vaccines

4.2.1 ASC responses in volunteers immunized with the oral Ty21a vaccine

Before vaccination, none of the volunteers had any circulating ASC specific to *S.* Paratyphi A/B/C, *S*. Typhimurium, *S*. Agona, *S*. Stanley, *S*. Virchow, or *Yersinia enterocolitica.* One volunteer had 5 ASC/10^6 PBMC reactive with *S.* Enteritidis and another 40 against *S.* Hadar before the vaccination (Figure 11). Seven days after vaccination cross-reactive plasmablast responses were examined. The numbers of the responders and numbers of the circulating ASC cross-reactive with paratyphoid and NTS serotypes are shown in Table 8 and in Figure 11. The arithmetic and geometric means, medians, 95% confidence interval (CI) and range of the numbers of cross-reactive ASC in all volunteers are shown in Table 8. Statistical comparisons between the responses against different serotypes/antigens are shown in Table 9 for the 25 volunteers immunized with the oral Ty21a vaccine. The cross-reactive responses were mostly dominated by IgA and IgM (data shown in the original article of Studies II and III).

4.2.2 ASC responses in volunteers immunized with the Vi vaccine

Before the vaccinations, no ASC specific to S. Paratyphi A/B/C or any of the NTS serotypes were found in the circulation of 24/25 vaccinees. One volunteer, having had one week earlier an upper respiratory tract infection, had 98 ASC/10^6 PBMC to *S.* Paratyphi A, 95 to B and 60 to Paratyphi C, 118 to *S.* Stanley and 95 to *S*. Hadar (Figure 12). All antigens were not measured for this volunteer before the vaccination. Seven days after vaccination cross-reactive plasmablast responses were examined. The numbers of responders, the proportion of vaccinees with response higher than 50 ASC/10^6 PBMC and the numbers of the circulating ASC cross-reactive with paratyphoid and NTS serotypes are shown in Table 9 or in Figure 12. The statistical comparisons between the responses against different serotypes are shown in Table 9. The cross-reactive responses were equally presented by IgA-, IgG- and IgM- ASC (data shown in the original articles of the Studies V and VI).

Table 8. Plasmablast responses to various *Salmonella* serotypes and negative control after immunization with the oral Ty21a vaccine. The numbers of volunteers, percentages of the responders, magnitude of the response to each NTS serotype in percentages of the *S*.Typhi-specific response, the number of plasmablasts (ASC/106 PBMC) specific to each strain are shown for volunteers vaccinated one week earlier with the oral Ty21a vaccine (arithmetic and geometric mean, median and 95% CI and min and max). Results with no specific ASC were replaced with value the 1 to enable calculation of geometric means.

		Plasmablast response					
Bacterial strain	**N**	**Percent of responders**	**Percent of response to *S.* Typhi**	**Arith. mean**	**Geo. mean**	**Median**	**95% CI (Min-Max)**
***S.* Typhi**	43	100	–	424	222	260	278-571 (15-1845)
***S.* Paratyphi A**	38	89	29	123	33	27	57-189 (0-851)
***S.* Paratyphi B**	43	93	43	184	59	64	89-278 (0-1449)
***S.* Paratyphi C**	43	21	1	3	2	1	2-4 (1-24)
***S.* Enteritidis**	43	100	100	354	190	226	239-468 (8-1402)
***S.* Typhimurium**	43	95	46	196	58	82	100-292 (1-1531)
***S.* Agona**	38	95	42	179	44	62	83-296 (1-1178)
***S.* Stanley**	38	92	45	191	44	53	86-296 (1-1366)
***S.* Virchow**	38	24	0	1	1	1	2-5 (1-19)
***S.* Hadar**	38	18	0,6	2,6	2	1	1-4 (1-24)
Yersinia enterocolitica	35	0	0	1	1	1	1-1 (1-2,5)

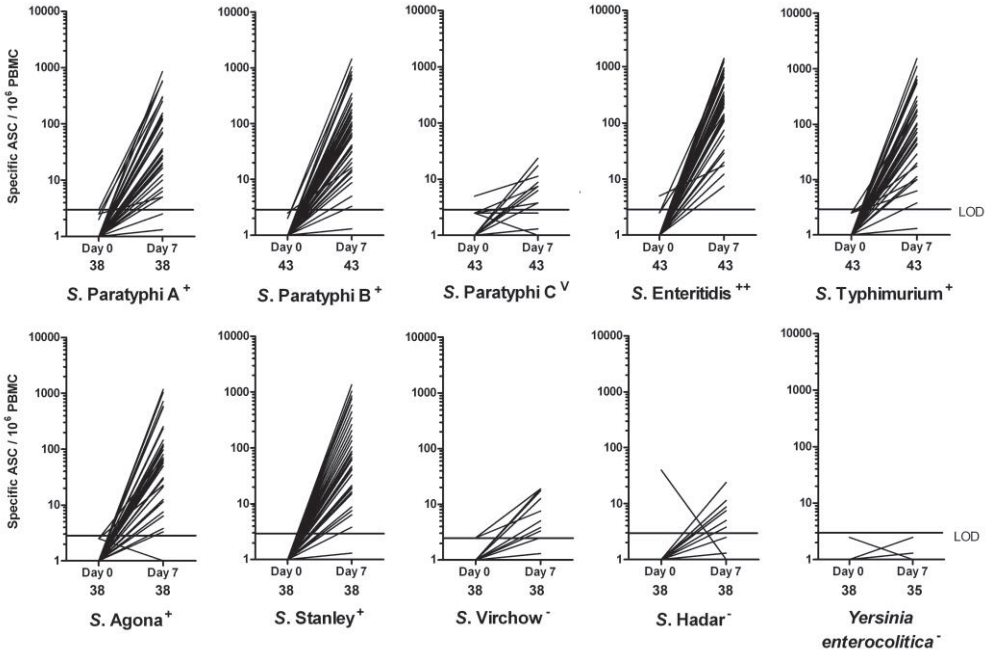

Figure 11. Plasmablast responses cross-reactive with various *Salmonella* serotypes and with *Yersinia enterocolitica* before and on 7 after immunization with the Ty21a vaccine. Numbers of circulating cross-reactive specific plasmablasts, identified as ASCs against paratyphoid and NTS serotypes and to the negative control *Yersinia enterocolitica* in volunteers immunized with the oral *Salmonella* Typhi Ty21a vaccine. The lines connect the numbers of Ig(A+G+M) plasmablasts of individual vaccinees on days 0 and 7 after vaccination. The number of volunteers for whom the data were pooled are indicated under the data bars. LOD = lower limit of detection (of the response). The upper indexes +/– indicate serotypes with (–) no O antigen, (+) one O-antigen (O-12) or (++) two O-antigens (O-9 and O-12) and V as Vi antigen in common with *S.* Typhi.

4.2.3 Comparison of cross-reactive ASC responses between the oral Ty21a and Vi groups

The number of responders and percentage of high responders among the oral Ty21a and Vi vaccine groups of 25 age-and gender-matched volunteers are shown in Table 9 and the statistical comparisons between the cross-reactive responses are provided in the same table. All of the cross-reactive responses to paratyphoid and to NTS serotypes expressing O-9 or O-12 or O-9,12 were higher in volunteers immunized with Ty21a than with the Vi vaccine (Table 9).

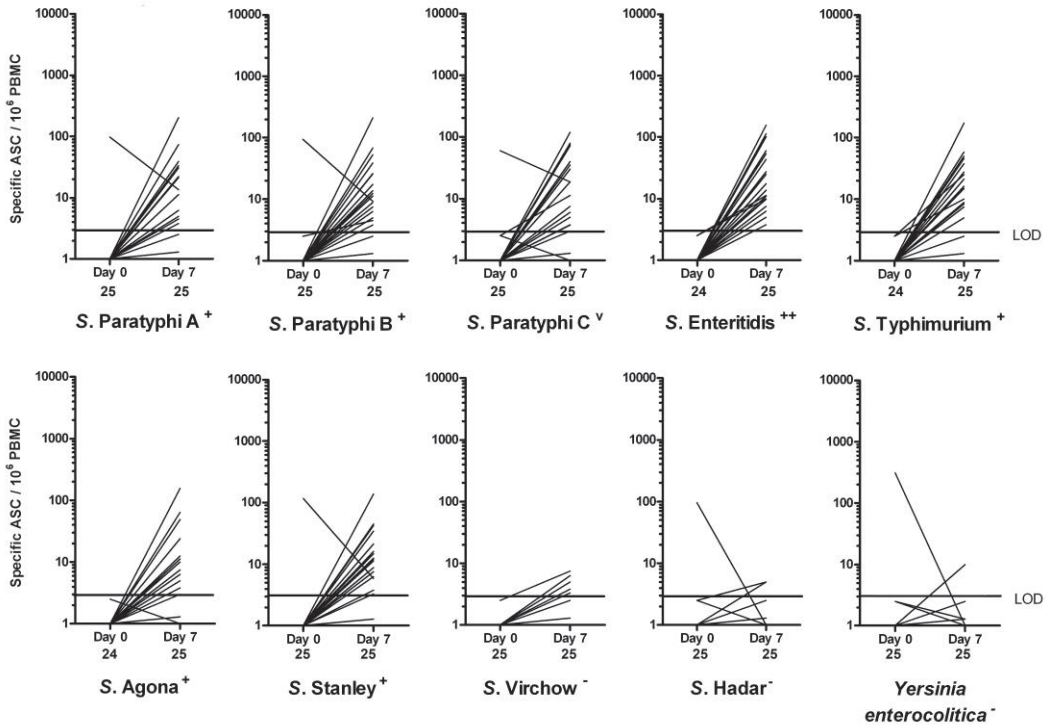

Figure 12. Plasmablast responses cross-reactive with various *Salmonella* serotypes and with *Yersinia enterocolitica* before and on 7 after immunization with the Vi vaccine. Numbers of circulating cross-reactive plasmablasts, identified as ASC against paratyphoid NTS serotypes and to the negative control, *Yersinia enterocolitica* in volunteers immunized with the Vi capsular polysaccharide vaccine. The lines connect the numbers of Ig(A+G+M)-plasmablasts of individual vaccinees on days 0 and 7 after vaccination. The number of volunteers from whom the data were pooled are indicated under the data bars. LOD = lower limit of detection (of the response). The upper indexes +/– indicate strains with (–) no O antigen, (+) one O-antigen (O-12) or (++) two O-antigens (O-9 and O-12) and V as Vi antigen in common with *S.* Typhi.

In both groups the highest responses were found against *S.* Enteritidis (expressing both typhoidal O-antigens, O-9,12). Significant responses were also seen to serotypes sharing one O-antigen with *S*. Typhi (O-12). The Vi vaccine elicited a response to *S*. Paratyphi C in contrast to the Ty21a vaccine (Table 9).

Table 9. Specific and cross-reactive plasmablast responses. Number of responders, percentage of vaccinees with response higher than arbitrary limit of 50 ASC/10^6 PBMC and numbers of antigen-specific ASC in 25 age-and gender-matched volunteers vaccinated with the oral Ty21a or the Vi vaccine (arithmetic means and 95% confidence intervals), statistical comparison (Wilcoxon signed-rank test without Bonferroni correction between the responses to various antigens and Wilcoxon signed-rank test in comparisons between vaccine groups). Significant differences are indicated with asterisks (*** p<0.001; ** 0.001<p<0.01; * 0.01<p<0.05). NS=not significant. Response to *S*. Typhi is calculated by sum of the response to O-9,12-H-d and Vi antigen).

Bacterial strain	Responders, [% of vaccinees with response higher than 50 ASC/10^6 PBMC]		Magnitude of plasmablast response			
			Arithmetic mean (95%CI) ASC/10^6 PBMC		Percent of response *S.* Typhi	
	Ty21a	Vi	Ty21a	Vi	Ty21a	Vi
***S.* Typhi**	25 [88]	25 [74]	339 (155-521)	149 (81-217)	-	-
***S.* Paratyphi A**	22 [39]	15 [8]	111 (31-197)	20 (2-37)	34	13
***S.* Paratyphi B**	23 [65]	18 [12]	137 (39-236)	22 (4-39)	40	15
***S.* Paratyphi C**	9 [0]	16 [16]	4 (1-6)	22 (8-35)	1	15
***S.* Enteritidis**	25 [86]	24 [28]	364 (235-493)	38 (19-55)	100	26
***S.* Typhimurium**	23 [60]	19 [12]	222 (105-339)	22 (8-37)	65	15
***S.* Agona**	23 [48]	16 [8]	191 (86-319)	17 (3-30)	56	11
***S.* Stanley**	23 [36]	18 [4]	205 (92-319)	16 (4-28)	60	11
***S.* Virchow**	9 [0]	8 [0]	2 (0-3)	2 (1-3)	0,6	1
***S.* Hadar**	6 [0]	2 [0]	1 (1-2)	1 (1-1)	0	1
Yersinia enterocolitica	0 [0]	1 [0]	0 (0-1)	1 (1-2)	0	0

Comparisons of the ASC responses
light gray: the Ty21a group
dark gray: the Vi group
white: the Ty21a vs. the Vi group

Sum of typhoid antigens	***S.* Paratyphi A**	***S. Paratyphi B***	***S. Paratyphi C***	***S.* Enteritidis**	***S.* Typhimurium**	***S.* Agona**	***S.* Stanley**	***S.* Virchow**	***S.* Hadar**	***Yersinia enterocolitica***
NS	***	***	***	*	***	***	***	***	***	***
***	*	*	***	***	NS	NS	NS	***	***	***
***	NS	*	***	***	NS	NS	*	***	***	***
***	NS	NS	*	***	***	***	***	NS	NS	**
***	***	**	**	***	***	***	***	***	***	***
***	*	NS	NS	**	*	NS	**	***	***	***
***	NS	NS	NS	***	*	**	NS	***	***	***
***	NS	NS	NS	***	*	NS	*	***	***	***
***	**	***	***	***	***	**	***	NS	NS	**
***	***	***	***	***	***	***	***	*	*	**
***	***	***	***	***	***	***	***	*	NS	NS

4.2.4 Cross-reactive antibody levels in ALS and serum samples

The cross-reactive responses elicited by the oral Ty21a and Vi vaccines were measured with three different approaches: the ELISPOT assay, which measures at a single-cell level plasmablasts producing antibodies; the ELISA assay for serum antibody responses and the ELISA assay for antibody levels in cell cultures (ALS).

The percentage of vaccinees with a twofold or higher increase in antibody titers in serum and ALS samples are shown in Table 10. Both vaccines elicit cross-reactive responses in serum as well as other samples. Number of immunized volunteers responding in the ELISPOT assay was higher than of that responding in the serum and ALS samples (Table 10).

Correlation analysis of the three different assays was performed on the Ty21a group in Study III. A correlation was observed between the magnitudes of the IgA responses in ALS and ELISPOT assays (*S.* Enteritidis: $r=0.9665$, $p<0.01$; *S*. Typhimurium: $r=0.9837$, $p<0.05$) and between the IgM responses to *S*. Enteritidis ($r=0.3809$, $p<0.05$), but not between the weak responses to *S*. Typhimurium. No correlation was found between the IgG responses in the ALS and ELISPOT assays or between the responses in the plasmablast assays and serum antibody assays with any Ig isotype investigated.

4.3 S. Typhi-specific and cross-reactive plasmablast responses in the patients with enteric fever

All patients with typhoid fever had circulating ASC specific to *S.* Typhi, *S.* Paratyphi A, B, C and *S*. Enteritidis, *S*. Typhimurium, *S*. Agona and *S*. Stanley. ASC specific to *S.* Egusi (H-d antigen), *S*. Virchow, *S*. Hadar and *Yersinia enterocolitica* were all either low in number or absent. The specificity of the response in a patient with paratyphoid A fever resembled that in patients with typhoid fever, except no response was mounted against *S*. Paratyphi C. This is consistent with the general view that *S*. Paratyphi A does not share antigens with *S*. Paratyphi C, while S. Typhi and S. Paratyphi C carry the Vi antigen. The number of pathogen-specific ASC in each patient and the means of the responses in patients with primary typhoid fever are provided in Figure 13.

Table 10. Cross-reactive immune responses to Ty21a and Vi vaccines evaluated with the aid of assays for antibodies in serum and in ALS and ELISPOT for plasmablasts. Percentages of the vaccinees responding in serum, ALS culture and ELISPOT assays to *S.* Enteritidis and *S*. Typhimurium in 25 age-and gender-matched volunteers vaccinated one week earlier with the oral Ty21a or the Vi polysaccharide vaccine. In the ELISA assays (serum and ALS), a responder was defined as an individual with at least a twofold increase in titer (in IgA, IgG andor IgM isotype). In the ELISPOT assay, a responder was defined as having at least 3 ASC/10^6 PBMC on day 7. Blood samples for ELISPOT and ALS were collected on days 0 and 7, and serum samples on days 0 and 28 after Ty21a vaccination. The number of vaccinees tested with each assay is indicated in the table.

	Responders (%) in the assay for											
	Serum antibodies/ELISA n=25				**ALS/ELISA** Ty21a: n=12, Vi: n=11				**ASC/ELISPOT** n=25			
	IgA	IgG	IgM	IgA/IgG/IgM	IgA	IgG	IgM	IgA/IgG/IgM	IgA	IgG	IgM	IgA/IgG/IgM
Oral Salmonella Typhi Ty21 vaccine												
***S*. Enteritidis**	24	24	44	60	92	25	50	84	92	76	92	100
***S*. Typhimurium**	12	4	20	24	50	17	25	50	72	64	92	92
Vi polysaccharide vaccine												
***S*. Enteritidis**	28	24	28	44	18	0	9	27	68	44	56	96
***S*. Typhimurium**	20	16	4	32	9	0	0	9	56	32	40	76

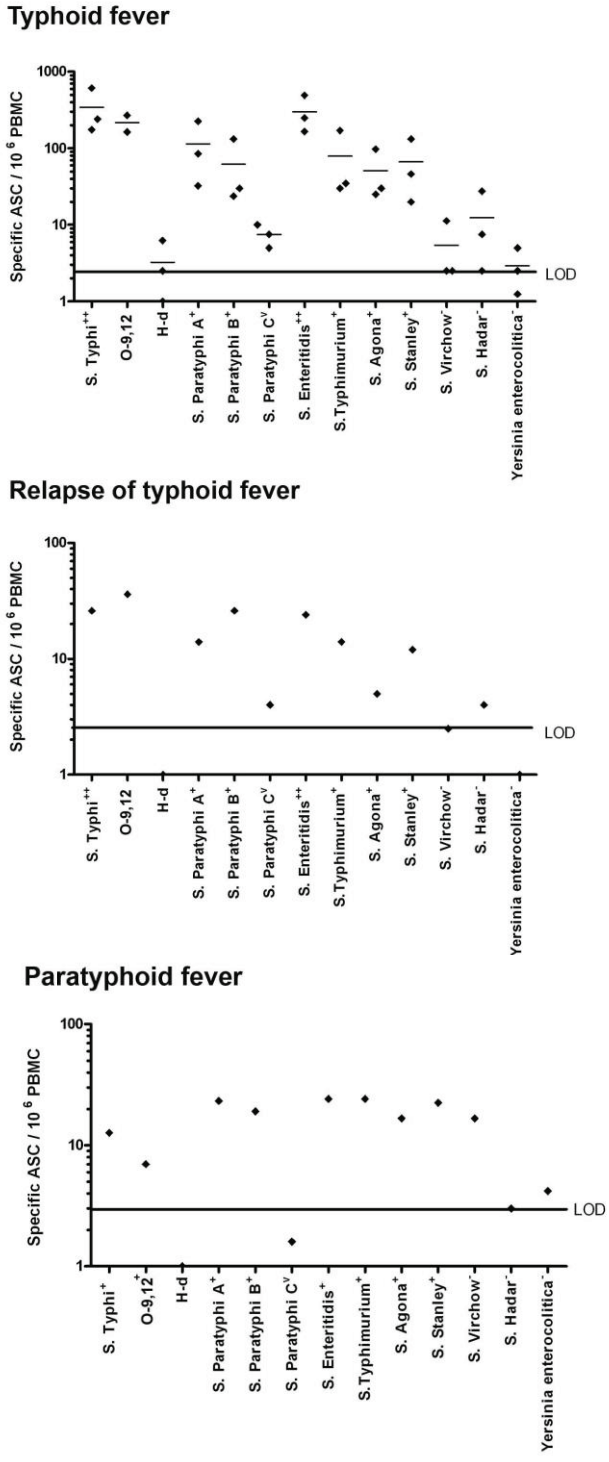

Figure 13. Pathogen-specific and cross-reactive plasmablast responses in patients with enteric fever. Numbers of circulating antigen-specific plasmablasts, identified as ASC in three patients with typhoid fever in the first panel, one patient with relapsing typhoid fever in the second panel, and one patient with paratyphoid A fever in the third panel. The dots represent the numbers of specific ASC in patients, and the lines the arithmetic mean of Ig(A+G+M) ASC/10^6 PBMC on day 7-10 after the onset of infection symptoms. LOD = lower limit of detection of the response. + indicates one O-antigen in common with the pathogen causing the disease (O-12), ++ indicates two O-antigens in common with the pathogen causing the disease (O-9 and O-12), V indicate Vi antigen common with the pathogen causing the disease.

4.4 Proportions of S. Typhi-specific or cross-reactive ASC expressing homing receptors $\alpha_4\beta_7$, L-selectin and CLA

4.4.1 Volunteers immunized with the oral Ty21a vaccine

The proportion of *S*. Typhi, *S*. Paratyphi B or *S*. Enteritidis-specific ASC expressing HRs $\alpha_4\beta_7$, L-selectin and CLA were determined in 16 volunteers immunized with the oral Ty21a vaccine. Altogether 95%, 97% or 98% of plasmablasts specific to *S*. Typhi, *S*. Paratyphi B, and *S*. Enteritidis, respectively, were found to express the intestinal HR, $\alpha_4\beta_7$, and 27%, 49% or 48% of them expressed the lymph node HR, L-selectin, and 4%, 7% or 8% the cutaneous HR, CLA, respectively (Figure 14). No differences existed between the HR expressions of plasmablasts specific to *S*. Typhi, *S*. Paratyphi A or *S*. Enteritidis (Figure 14). In addition, no differences were seen in HR expressions in plasmablasts secreting IgA1 or IgA2 specific to *S*. Typhi (Figure 15).

Homing properties of ASCs elicited by the oral Ty21a vaccine resembled those elicited by natural infections caused by *S*. Typhi or *S*. Paratyphi A (see Section 4.4.3 and Figure 16, the numbers of cases were too low to allow statistical comparisons.) By contrast, the homing profile differed from that found after parenteral immunization with Vi vaccine (see Section 4.4.2 and Figure 14).

4.4.2 Volunteers immunized with the Vi vaccine

In 12 volunteers immunized with the Vi vaccine, the proportion of *S*. Typhi or *S*. Enteritidis-specific ASC expressing HRs $\alpha_4\beta_7$, L-selectin and CLA were determined. Of these plasmablasts, 56% and 61% were found to express $\alpha_4\beta_7$, 84 % and 77 % L-selectin, and, 4 % and 6 % CLA, respectively (Figure 14). No differences were observed between HR expression on plasmablasts specific to *S*. Typhi or *S*. Enteritidis (Figure 14).

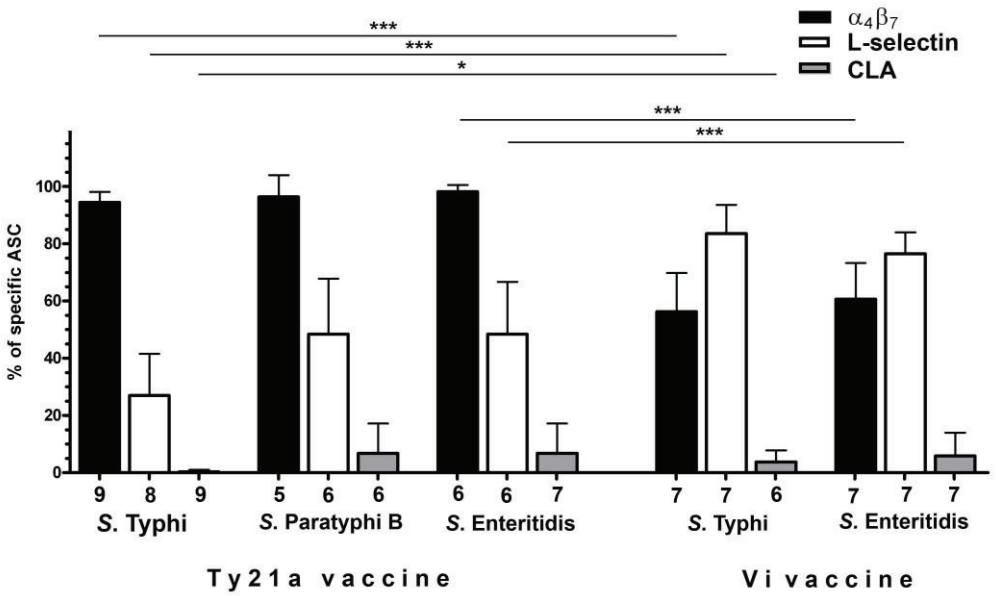

Figure 14. Homing potentials of the *S.* Typhi-specific and cross-reactive plasmablasts in volunteers immunized with the Ty21a or Vi vaccine. Percentage of ASC in homing receptor positive $\alpha_4\beta_7$, L-selectin and CLA on *S.* Typhi, *S.* Paratyphi B (only the Ty21a-vaccinated volunteers), and *S.* Enteritidis -specific plasmablasts in the peripheral blood of volunteers immunized one week earlier with the oral Ty21a or parenteral Vi vaccine. The bars indicate the arithmetic means + 95%CI of percentages of HR-positive ASC among all antigen-specific ASC (IgA+IgG+IgM). The HR and the numbers of volunteers for whom the data were pooled are indicated under the data bars. The statistical comparisons (Wilcoxon signed-rank test) between the two vaccination groups are indicated with asterisks (*** p<0,001; ** 0.001 < p < 0.01, * p<0,05). There were no differences in the expression of the HR between the plasmablasts specific to different bacterial serotypes within the vaccination groups.

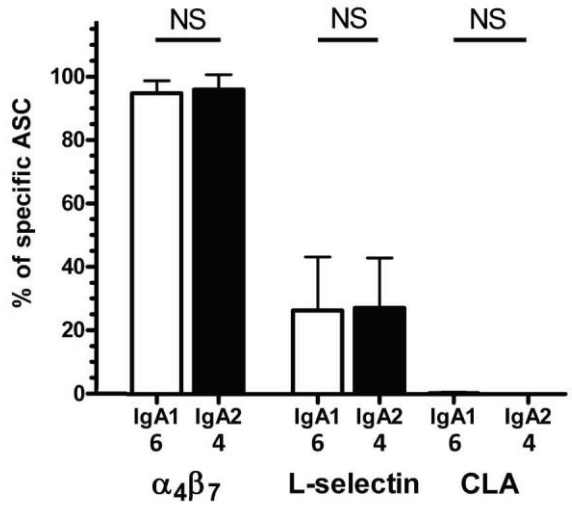

Figure 15. Homing potentials of *S.* Typhi-specific ASCs in volunteers immunized with the oral Ty21a vaccine. The arithmetic means (±SD) of the percentages of *S.* Typhi-specific IgA1- and IgA2-secreting cells expressing HR in volunteers immunized one week earlier with the oral Ty21a. The numbers of volunteers for whom the data were pooled are given under each bar. The statistical comparisons are performed with paired Student's-test. NS=not significant.

4.4.3 Patients with enteric fever

The proportions of cells expressing $\alpha_4\beta_7$, L-selectin and CLA among ASCs reactive with the pathogen in patients with enteric fever were determined. A high proportion of ASCs expressing $\alpha_4\beta_7$ was seen both in patients with typhoid fever (91%) and in the patient with paratyphoid fever (94%). The proportion of ASC expressing L-selectin (40% in typhoid fever and 77% in paratyphoid fever) or CLA (11 % and 0%, respectively) were lower than those expressing a4b7. The patient with relapsing typhoid fever had a high proportion of ASC expressing all three HRs ($\alpha_4\beta_7$ 95%, L-selectin 66 %, CLA 65%) (Figure 16).

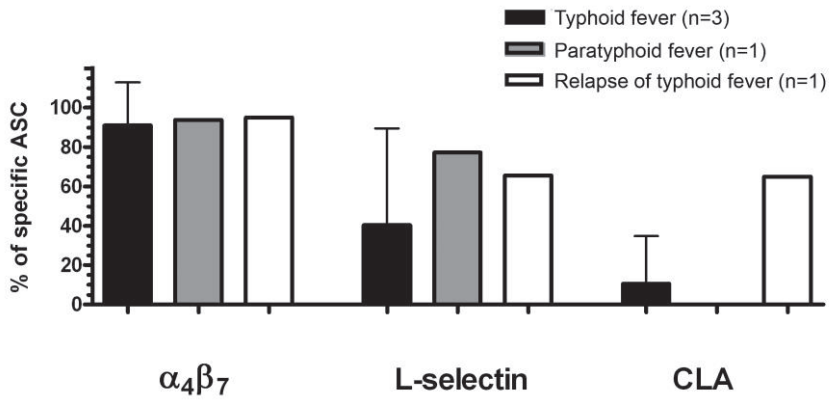

Figure 16. Homing potentials of the disease causing pathogen-specific plasmablasts in patients with typhoid fever, paratyphoid fever or relapsing typhoid fever. The expression of $\alpha_4\beta_7$, L-selectin and CLA on *S.* Typhi- or *S*. Paratyphi A-specific plasmablasts in the peripheral blood of patients with typhoid fever (n=3), paratyphoid A fever (n=1) or relapsing typhoid fever (n=1) seven to ten days after onset of infection. The bars indicate the arithmetic means + 95%CI of percentages of HR-positive ASC among all pathogen-specific ASC (IgA+IgG+IgM).

5 DISCUSSION

5.1 Immune response to S. Typhi

5.1.1 *S.* Typhi-specific ASC response elicited by the Ty21a or the Vi vaccine

The studies included in this thesis are the first to compare head-to-head the immune responses generated by the two commercially available typhoid vaccines, the Ty21a and the Vi preparation. Both vaccines induced similar numbers of typhoid-specific plasmablasts in the circulation of vaccinees, consistent with the similar efficacies of these vaccines in field trials.[150, 198, 204] The Ty21a vaccine elicited strong responses to the O-9,12 antigens and no response to Vi antigen, in line with antigens expressed on the surface of the vaccine strain. The Vi vaccine contains purified Vi antigen and, accordingly, elicits a plasmablast response to Vi antigen. Surprisingly, however, a response was also elicited to typhoidal O-antigens; this was presumably due to traces of lipopolysaccharide remaining in the vaccine preparation. Consistent with differences in the magnitude of O-antigens in the preparation, the O-antigen-specific responses were lower among the Vi- than the Ty21a-vaccinated volunteers. A Vi antigen-specific response was seen only in the Vi group. As far as we know, our study was the first to show O-antigen- or even Vi antigen-specific plasmablasts following immunization with the Vi vaccine in humans. Recently, Marhall et al. have reported Vi antigen-specific ASCs after immunization with Vi vaccine in mice,[240] and Wahid et al. have demonstrated Vi antigen-specific memory B cells in humans after immunization with the Vi vaccine primed with oral *Salmonella* Typhi vaccine expressing Vi antigen (CDV 909).[116] *S*. Typhi or *S*. Typhi LPS-specific ASCs have been shown in numerous previous studies after the Ty21a vaccine[19, 20, 23, 26, 62, 69, 119] or the other oral attenuated typhoid vaccine.[241]

No immune response was detected to the H-d antigen in either of the groups. A previous study by Kantele et al.[62] reported a plasmablast response against H-d antigen after immunization with the parenteral whole-cell Ty21a, but not after oral Ty21a vaccination,[62] suggesting that immunization route influences processing of

the antigen. The fact that no response was found in the Vi group against H-d suggests that either the H-d antigen was destroyed when Vi antigen was purified or the amount in the preparation has remained too low to elicit a measurable immune response.

Comparison of the homing properties of antigen-specific ASC revealed significant differences between the two vaccines. Specific plasmablasts elicited by the oral Ty21a vaccine had an intestinal homing profile (majority of the cells expressing the intestinal HR $\alpha_4\beta_7$, and a lower proportion the peripheral lymph node HR, L-selectin and cutaneous HR, CLA), while specific plasmablasts after immunization with the Vi vaccine had more systemic homing properties (higher proportion of L-selectin$^+$ cells, lower of $\alpha_4\beta_7^+$ and CLA$^+$ cells). These data are consistent with previous reports that the HR profile depends on the site where the antigen is encountered.[80, 121] The present study was the first to examine the homing properties after immunization with the Vi vaccine; the systemic homing profile is consistent with the parenteral administration route of the vaccine.

In addition to the group receiving oral Ty21a or parenteral Vi vaccines, *S*. Typhi-specific ASC responses were also found in groups immunized with the rectal and parenteral Ty21a vaccines. In the original article of Study I, data on IgA were shown, yet in this thesis (Figure 10) the results are shown not only for IgA but also for IgG and IgM isotypes. The magnitude of the response elicited by the oral Ty21a vaccine was similar to that after rectal and higher than that after the parenteral Ty21a vaccine, consistent with earlier studies.[62, 119]

5.1.2 Measuring *S*. Typhi-specific responses with ALS and ELISPOT assays and serum antibodies in volunteers immunized with typhoid vaccines

We compared the responses to Ty21a and Vi vaccines by three different approaches: by analyzing the numbers of ASCs with ELISPOT and by determining the levels of secreted antibodies in serum and ALS samples with ELISA. The serum antibodies measure systemic humoral immune responses, while the ELISPOT and the ALS assays both measure either mucosal or systemic plasmablast responses. ELISPOT provides information on the relative proportion of specific plasmablasts (ASCs) among all plasmablasts (ISCs) (or among PBMCs) while ALS describes

amounts of antibodies produced by these plasmablasts. The findings in the ELISPOT and ALS assays should always be related to the HR profiles of the plasmablasts; after oral immunization they mostly describe the responses in the mucosal immune compartment, while after parenteral vaccination they provide mostly information on systemic immune responses. The ALS assay proved to be more sensitive than the serum antibody assay in the Ty21a group but not in the Vi group. This is consistent with the gut-derived immune response elicited by the oral Ty21a and the more systemic response in the parenteral Vi group. These data nicely record the homing profiles of plasmablasts in these groups. Consistent with previous studies, the ELISPOT assay measuring antibody production at a single-cell level was found to be more sensitive than the ALS assay[26] or the serum antibody assay[23, 26] in both vaccine groups.

5.2 S. Typhi-specific ASC response elicited in natural typhoid fever

S. Typhi-specific plasmablast responses in patients with typhoid fever have earlier been shown with the ALS.[242, 243] *S*. Typhi-specific serum antibodies are widely used in diagnostics of typhoid fever (Widal test). *Salmonella*-specific ASCs elicited by a natural infection have previously been studied after NTS gastroenteritis.[56, 66] This thesis, it was demonstrated for the first time that pathogen-specific ASCs appear in the circulation in a natural infection caused by *S*. Typhi or *S*. Paratyphi. Unfortunately, the number of volunteers enrolled remained low, as the cases in Finland are so limited (approximately 20/year).

Responses to *S*. Typhi were substantial in typhoid fever (175, 241, 612 ASC/10^6 PBMC in primary infection), but lower (27 ASC/10^6 PBMC) in the single case with relapsing typhoid fever. Even if no definite conclusions can be drawn with such a low number of volunteers, the data may suggest that a lower ASC response could be associated with relapses: the primary infection induces antibodies in the intestine and such pre-existing antibodies could work against the re-infection by preventing bacterial contact with the intestinal epithelium as previously suggested in human experiments on oral Ty21a vaccine.[229, 230, 244] The same reasoning could apply to the response in the patient with paratyphoid fever; her fairly low plasmablast response mightbe due to the fact that this was an Indian patient who

potentially could had pre-existing (cross-reactive) intestinal antibodies due to a previous contact with *S*. Paratyphi or other related *Salmonella* serotypes.[229, 230, 244]

The HR profiles of the specific plasmablast in natural typhoid or paratyphoid fever indicate that these cells preferentially migrate to the intestine. In previous studies, similar gut-directed HR profiles have been reported in patients with NTS gastroenteritis[79] and in infections caused by other intestinal pathogens[79] or after oral immunization with the Ty21a[80, 121] and the cholera vaccine.[121] Notably, the homing profile in the patient with relapsed typhoid fever with a high expression of all HR studied resembled that described previously after booster immunization with the oral Ty21a vaccine[226] and that in natural infection of *Vibrio cholerae* in an endemic area, Bangladesh.[245]

5.3 Cross-reactive plasmablast responses

5.3.1 Immunological basis for cross-reactivity

The immunological basis for the cross-reactive response against *S*. Paratyphi A and B and against certain NTS types can be explained by the O-antigens shared between the oral vaccine strain (Ty21a) or the parenteral preparation (the Vi vaccine) and their serotypes. Ty21a is a whole-cell vaccine expressing the typhoidal O-9 and O-12-antigens, while the Vi preparation includes these antigens only as it contains trace amounts of typhoid LPS that have remained after purification. *S*. Paratyphi A and B both express the O-12 antigen. Some of the NTS serotypes share both typhoidal O-antigens (e.g. *S*. Enteritidis) while others share only the O-12 antigen (e.g. *S*. Typhimurium) or none at all (e.g. *S*. Hadar). The cross-reactive response against *S*. Paratyphi C found after immunization with the Vi vaccine and in natural typhoid fever is presumably due to the fact that *S.* Paratyphi C carries the Vi antigen present in the vaccine and in the wild type *S*. Typhi.

5.3.2 Cross-reactive ASC responses elicited by the Ty21a or Vi vaccine

The magnitude of all cross-reactive responses to paratyphoid A and B and NTS serotypes proved significantly higher in the Ty21a than the Vi group. The cross-

reactive responses to serotypes with only one typhoidal O-antigen were significantly lower than those to *S.* Enteritidis or *S*. Typhi (both carry O-9,12) in both vaccination groups. In the Ty21a group, in more than 50% of the vaccinees the magnitude of the response to *S*. Typhi, *S*. Paratyphi B, *S*. Enteritidis and *S*. Typhimurium exceeded 50 ASC/10^6 PBMC. In the Vi group, by contrast, none of the volunteers reached this level of cross-reactivity.

The homing profiles of the cross-reactive plasmablasts were tested against *S*. Paratyphi B and *S.* Enteritidis after immunization with the Ty21a vaccine and against *S*. Enteritidis after the Vi vaccine. The homing profiles were found similar to those of *S*. Typhi-specific plasmablasts in the same volunteers (intestinal homing after oral vaccination and systemic after parenteral vaccination). This finding was not surprising because these cells are presumably the same vaccine strain-specific cells showing cross-reactivity.

5.3.3 Measuring cross-reactivity with ELISPOT assays for plasmablast and ELISA for antibodies in ALS and serum samples in volunteers immunized with typhoid vaccines

Cross-reactivity of typhoid vaccines was investigated by two plasmablast assays: ELISPOT assay (results discussed above) and ALS assay as well as by measurement the serum antibodies. All these methods supported cross-reactivity. These assays showed a response more frequently to *S*. Enteritidis than to *S*. Typhimurium, in line with the number of O-antigens shared.

5.3.4 Cross-reactive ASC response elicited in natural enteric fever

The cross-reactive ASC responses were similar in those immunized with the Ty21a vaccine and those with natural enteric fever. This was observed with both paratyphoid A and B serotypes as well as several NTS serotypes. The only exception to this was the response to *S*. Paratyphi C; it could only be detected in those with typhoid fever, not in the vaccinees. This is consistent with the fact that *S*. Paratyphi C does not sharing O-antigens with *S*. Typhi nor with the Ty21a strain, yet it expressing the Vi antigen found on the wild-type *S*. Typhi strains of these patients, but not in the attenuated Ty21a strain. Expectedly, the response in

paratyphoid fever was lower against *S*. Typhi than to *S*. Paratyphi, presumably because of the fact that *S*. Typhi and the disease causing pathogen only share one common O-antigen (O-12).

5.4 Protective capacity of O-antigen-specific antibodies

The cross-reactive immune responses shown in the present study appear mostly to be directed against the O-antigen, thus making the cross-protective capacity of O-antigen-specific antibodies a central issue. The reports in the literature describing the cross-protective capacity of the Ty21a vaccine against paratyphoid fever are somewhat conflicting. The studies of cross-reactivity or cross-protection against paratyphoid serotypes are described in more detail above in Sections 1.10.3 and 1.10.4 in the literature review. In brief, Levine et al. have shown, by re-visiting a large field trial with the oral Ty21a vaccine, that this vaccine provides a protective rate of 58% against typhoid fever and simultaneously conferring 49% cross-protection against *S*. Paratyphi B.[22] By contrast, no cross-protective efficacy against *S*. Paratyphi A was found in another large field trial by Simanjuntak et al.[218] However, in that trial an abnormal vaccination protocol was used and also the protection against typhoid fever proved poor; an efficacy of 42% was reported with the enteric-coated capsule. On the other hand, Meltzer et al. investigated the incidence of enteric fever in Israeli travellers receiving Ty21a or Vi vaccines and concluded that the Ty21a vaccine may confer some cross-protection against *S.* Paratyphi A.[142] In addition, Schwartz et al suggested a 72-75% efficacy against *S*. Paratyphi A by the TAB vaccine (most travellers received TAB, only a minority received Ty21a). By contrast, Bodhidatta et al. reported no protective capacity against *S*. Paratyphi A with TAB.[220] In addition to the field trials listed above, there are several immunological studies describing cross-reactivity either with cell-medicated,[16-19] or IgA antibody-enhanced cell-medicated mechanisms,[16-19] as IgA- and IgG-ASC responses,[19] circulating memory cells,[19] or as opsonophagocytic activity correlating with levels of serum O-antigen-specific antibodies[21] against *S*. Paratyphi A and B. The results support the findings of this thesis.

Until this work, there have been no studies describing the cross-protective capacity of typhoid vaccines against NTS, apart from one paper noting such cross-reactivity in the validation process of the S. Typhi-specific ELISPOT assay.[63] The

data of the protective efficacy of the O-antigen specific antibodies against NTS have been reviewed above in Section 1.9.4 and the data of the function of these antibodies in Section 1.9.3. The role of the O-antigen specific responses in protection should be considered separately for mucosal and systemic antibodies and both with respect to NTS and enteric fever. Theoretically, mucosal antibodies are expected to contribute to protection against non-invasive NTS disease and during the intestinal phases of enteric fever, while systemic responses are expected to be of important in invasive NTS infections and in the post-intestinal phases of typhoid and paratyphoid fever. Maclennan et al. have shown in African children a negative correlation between the case fatality rate of iNTS and the level of O-antigen-specific serum antibodies.[184] Intestinal IgA antibodies against *S.* Typhimurium O-antigens have proven protective against invasive[181, 189, 190] or mucosal infection[191] in animals, as have intravenously administered O-antigens IgM antibodies against intraperitoneal *S*. Typhimurium challenge have proved protective[186, 188] By contrast, O-antigen-specific antigens were not found to protect against parenteral challenge with the bacteria.[186, 191]

5.5 Significance of cross-reactive immune response

The clinical significance of a potential cross-protection conferred by the Ty21a or Vi vaccines depends on the prevalence of *Salmonella* serotypes carrying the O-9 or O-12 antigens is in the region of interest.

Cross-protective potential against paratyphoid fever would have clinical significance only in regions where the disease is endemic. *S*. Paratyphi is common in many tropical and subtropical regions with an estimateed 5.4 million cases annually. The incidence is highest in the Indian subcontinent. *S*. Paratyphi C, by contrast, is a rare cause of paratyphoid fever, and has thus only limited clinical significance. *S.* Paratyphi A and B both express only one of the typhoidal O-antigens (O-12), and, accordingly, the cross-reactive response elicited by the vaccines is not very high in magnitude. This suggests that any cross-protective potential is also not expected to be very high or to reach the level needed against typhoid fever. This is consistent with the findings of MM Levine et al. showing a lower level of protection against *S*. Paratyphi B than against *S*. Typhi in Chile.[22]

Non-typhoid salmonellosis is a leading cause of food-borne infections in both developing and developed countries, with a total estimate of 90 million annual cases world-wide. *S.* Enteritidis (with both O-9 and O-12) and *S*. Typhimurium (O-12) are the causative agents in most of the iNTS and non-invasive salmonellosis cases.[129, 156, 157, 165, 169, 170] The cross-reactive response to *S*. Enteritidis appears especially promising as it equals in magnitude that to *S*. Typhi, consistent with these serotypes having both O-antigens in common.

5.6 Plasmablasts as surrogate markers for protection

The concept of using specific circulating plasmablasts as surrogate markers for protection against typhoid fever is based on previous findings[118] that their number increases[23] concomitantly with increasing efficacy of the same vaccine regimen in field trials.[123, 232] Potential protection offered by the vaccines should be evaluated in relation to the localization of the immune response, which can partly be studied by investigating the homing properties of the activated plasmablasts: oral vaccination induces plasmablasts with intestinal homing properties,[80, 81] and causes intestinal antibody production,[119, 222] while plasmablasts after parenteral vaccination elicit more systemic responses.[80, 121] Thus, homing properties of the specific plasmablasts reflect the localization of the response, which may affect the practical question whether the vaccine is conferring protection against local or systemic disease.

The use of plasmablasts as a surrogate marker for protection can be criticized because the field trials have been conducted in endemic areas, whereas plasmablasts have been assessed in volunteers from non-endemic areas:[23] in contrast to travelers, inhabitants of endemic areas may have some degree of pre-existing immunity due to potential previous encounters with *S.* Typhi, *S.* Paratyphi or O-9,12 or O-12 positive non-typhoid *Salmonella* serotypes. Such pre-existing immunity can influence the intestinal immune response to anoral live vaccine in two alternative ways: (1) in those with active intestinal antibodies the vaccine bacteria may be excluded, thus leaving the vaccination with a weaker response or no effect at all[229, 230, 244] or (2) in those with less active immunological status yet with an immunological memory, the vaccine may boost the response, and a more vigorous intestinal immune response ensues.[231] In addition to pre-existing

immunity, inhabitants in some endemic areas may have suffered from malnutrition or loss of vitamin A, which could lead to impaired mucosal immune responses.[246] Differences in intestinal microbiota between industrialized and low-income countries could also have an impact.[114] A lower protective efficacy in endemic areas than in industrial countries is described as tropical barrier.[114] It has been suggested to be due to tropical enteropathy.[247] An example of this is the rotavirus vaccine which has a 30% lower protective efficacy in developing countries than in industrialized countries.[114, 248, 249] Indeed, the protective efficacy reported in the field trials in endemic areas should be extrapolated to travellers with some caution.

Hence, the plasmablast responses in non-endemic areas represent some kind of baseline responses, and these limitations should be kept in mind when applying the results in endemic areas. It should be pointed out, however, that field trials are very expensive and laborious to conduct and, therefore, despite the limitations described above, the plasmablast assay has proved helpful as a laboratory test when developing oral vaccines. It is considered to correlate better with protection than the other assays, and thus represent the best surrogate marker available at present. No surrogate markers for protection against paratyphoid fever or NTS have been suggested. However, it appears reasonable to think that if plasmablasts can be used as surrogate markers for protection against *S*. Typhi, the same approach could be used for these as well.

Even if plasmablasts could be used as surrogate markers of protection, a definite protective level does probably not exist, as it is well known that any immunity to Salmonella can be overcome if only the bacterial load is sufficiently high.[250]

5.7 Immunoglobulin isotype distribution of specific and cross-reactive ASC elicited by typhoid vaccines

In the majority of vaccinees, the immunoglobulin isotype distribution showed a predominance of IgA and IgM plasmablasts, irrespective of the type of typhoid vaccine administered. This is consistent with earlier studies showing that immunization by the oral route[23, 58, 62, 63, 251, 252] and with polysaccharide structures[107, 122] elicit IgA-dominant responses. Previously it has been

demonstrated with the oral Ty21a vaccine that the IgM response peaks on day 5, and IgG and IgA responses on day 7,[23] and the sum of these three (IgA+IgG+IgM-ASC) on day 7.[23] Furthermore, kinetics of the ASC response after oral, parenteral,[62] and rectal[119] immunizations with Ty21a have been shown to be similar.

Notably, the immunoglobulin class switch of mucosal IgA cells can take place only after their arrival in the lamina propria.[87] Accordingly, the IgM-secreting plasmablasts found in the blood may mature into IgA-producing cells even after migrating into the intestinal lamina propria. Therefore, it is important to analyze all three Ig-classes when assessing mucosal immune response with the help of circulating plasmablasts.

5.8 IgA subclass distribution

This thesis focused only on a small part of the results presented in Study I, the IgA subclass distribution of the responses after immunization with the Ty21a vaccines. The key question in study I was to determine whether the unequal distribution of IgA1 and IgA2 subclasses between various secretions is based on differential homing of IgA1 and IgA2 cells in the body or on something else.

The nature of the antigen had a profound effect on the IgA subclass distribution of the response: an IgA2-dominated response was found to polysaccharide antigens (pneumococcal polysaccharide) and an IgA1-dominated response to protein antigens (diphtheria toxoid), consistent with studies by other groups.[107-109]

The homing of IgA1- and IgA2-ASC was evaluated with help of HR expression and concentrations of these subclasses in various secretions. Previous studies by others have shown that the distribution of IgA1 and IgA2-B cells in the various mucosal tissues equals to the distribution of these subclasses in the respective secretions.[105, 106] Consistent with results of previous studies and those of the present study, the total IgA1 predominated in all other secretions but not the vaginal and the intestinal fluids; the only secretion in which IgA2 dominated was the intestinal fluid. The total IgA2-ISC in the circulation were found to express intestinal homing receptors more frequently than the IgA1-ISC suggesting that the differential homing of these cells would account for the differential distribution of

these subclasses between various secretions. However, ISC represent the total of various antigen-specific ASC and the HR profile could thus simply be influenced by the gut-derived plasmablasts (predominantly IgA2) being more abundant than plasmablasts from other sites (predominantly IgA1). To explore this possibility, a more detailed analysis of antigen-specific responses was warranted. Indeed, the nature of the antigen was found to determine the IgA subclass distribution of the response, with polysaccharide structures producing IgA2- and protein structures IgA1-dominated responses. The homing profiles of these antigen specific ASC were similar between IgA1- and IgA2-ASC of a given specificity. As an example, Ty21a-specific IgA1 and IgA2-ASC in the circulation had similar intestinal homing properties even if the *S.* Typhi-specific IgA1 predominated in all secretions and among Ty21a-specific ASC in the circulation. Likewise, a parenteral polysaccharide vaccine was found to produce an IgA2-dominated response yet with specific IgA1- and IgA2-ASC having an identical homing profile. Our data suggest that the site of antigen encounter determines the homing profile, but does not influence the IgA subclass distribution. This site-specific homing together with the nature of the antigens present at various sites determines the IgA subclass distribution at that given site. The intestinal homing along with the huge polysaccharide and LPS loads of Gram-negative bacteria in the lower intestine and the genital tract but not at other mucosal sites thus probably results in an influx of IgA2-dominated plasmablasts at these sites, and hence accounts for the unequal distribution of IgA subclasses in various secretions.

6 SUMMARY AND CONCLUSIONS

The present study demonstrates that the plasmablast responses to *S*. Typhi elicited by the oral Ty21a and Vi vaccines are similar in magnitude, but they differ in antigen specificity. Both of these vaccines elicited cross-reactive responses to numerous *Salmonella* serotypes, all of which share *S*. Typhi O-9 or O-12 antigens or both of these antigens. Similar cross-reactive responses were found in patients with enteric fever. Cross-reactive responses were induced to several clinically significant pathogens such as *S*. Paratyphi A and B, *S*. Enteritidis and *S*. Typhimurium, which cause a marked disease burden worldwide. These responses were found to be significantly higher in volunteers receiving the Ty21a than the Vi

vaccine, that response to the Vi vaccine presumably only based on contaminating lipopolysaccharide present in the preparation. The homing profiles of these plasmablasts were found to depend on the administration route; the Vi vaccine elicited mostly a systemic response and the oral Ty21a vaccine mostly an intestinal response. Plasmablast responses in natural enteric fever were found to resemble those elicited by Ty21a.

Plasmablast responses elicited by both Ty21a and Vi vaccines were mostly dominated by IgA and IgM. Furthermore, after immunization with the Ty21a vaccine, either orally, rectally or parenterally, specific IgA1-ASC predominated over IgA2-ASC in the circulation and the proportion of specific IgA1-antibodies was higher than that of IgA2 in all secretions (saliva, nasal lavage, tears and intestinal samples). *S*. Typhi-specific IgA1- and IgA2-ASC elicited by the oral Ty21a vaccine had similar intestinal homing profiles. The unequal distribution of total IgA subclasses between various secretions was concluded to depend on the nature of the antigens stimulating immune responses and not on the route of immunization.

The immunological findings clearly show a significant cross-reactive capacity against *S*. Paratyphi A and B and against numerous NTS serotypes, especially with the Ty21a vaccine. Cross-protection against *S*. Paratyphi B has already been confirmed in field trails; however, efficacy studies of cross-protectivity are required before final conclusions about protective efficacy against the other serotypes can be drawn. There are no vaccines available against NTS and *S*. Paratyphi, yet these pathogens constitute a significant global health problem, which is further complicated by the emerging antimicrobial resistance among all *Salmonella* serotypes. In the present situation, any degree of cross-protective capacity in a vaccine currently available should be welcomed and explored further.

Table 11. Summary of the central findings of this thesis. Magnitude of the response as evaluated with respect to the proportion of volunteers responding with more than 50 ASC/10^6 PBMC: ++++ indicates a proportion of 100-70%, +++ 69-40%, ++ 39-25%, and + 24-5%.

	Ty21a vaccine	**Vi vaccine**
S. Typhi (O-9,12 and Vi)	++++	++++
S. Paratyphi A **(O-12)**	++	+
S. Paratyphi B **(O-12)**	+++	+
S. Enteritidis **(O-9 and -12)**	++++	++
S. Typhimurium **(O-12)**	+++	+
Homing of vaccine specific cells	gut	systemic

7 FUTURE PROSPECTS

Enteric fever and non-typhoidal salmonellosis remain diseases with global significance despite improvements in the hygienic situation in developing countries; still 780 million people exist without clean water and 47% of the world's population does not have proper sanitation.[253] Enteric fever is mostly only seen in developing countries while diseases caused by NTS serovars are encountered also in high-income countries, where *Salmonella* outbreaks[10-12, 254] are often associated with industrialization of food production.[162] In developed countries, enteric fever is mostly only encountered in travellers.[8, 255]

The emergence of antimicrobial resistance among *Salmonella* strains poses a serious public health concern. The strains are transmitted via food, and may also be contracted from animals and infected persons. In this era of globalization, the strains are distributed not only locally but also to distant countries and continents. Travellers may also be at risk; the annual number of international arrivals exceeds one billion people.[256] The increasing antimicrobial resistance stresses the need for preventive measures, i.e. vaccination and improvement of hygienic control.[201, 257, 258]

New vaccines against *S*. Typhi are being developed:[201, 203] the conjugated Vi-[259-262] and the *S*. Typhi O-antigen-[263] based regimen and the various oral live attenuated *S*. Typhi vaccines.[116, 117, 264-266] The latter two of these new typhoid fever vaccine candidate types contain O-9,12 antigens and are therefore expected to elicit cross-reactive immune response against *S*. Paratyphi A and B[21] and also for NTS serovars sharing *S*. Typhi O-antigens, as seen in our results with the Ty21a vaccine. New vaccines are not only being developed against typhoid fever, but also against *S*. Paratyphi[267, 268] or NTS.[269-273] Several research groups are trying to develop vaccines to prevent iNTS. Various platforms have been proposed such as combination O-polysaccharide conjugates[269-271] and attenuated live oral vaccines.[268, 272, 273]

Until the new vaccines against *S*. Paratyphi and/or NTS serovars are approved, the Ty21a vaccine is the only vaccine that potentially can be expected to confer some protection against *S*. Paratyphi and certain NTS serotypes. The registration and wide use of this vaccine encourages further studies exploring this potential.

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